CNG2018 022

POLIMORFISMOS NULOS DE LOS GENES *GSTT1* Y *GSTM1* Y ENFERMEDAD DE PARKINSON

Alvarado-Retana KM, Salas-Pacheco SM, Antuna-Salcido EI, Sandoval-Carrillo AA, Castellanos-Juárez FX, Méndez-Hernández EM, La Llave-León O, Salas-Pacheco JM*

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La enfermedad de Parkinson (EP) es una patología neurodegenerativa que afecta aproximadamente al 3% de la población mundial, es multifactorial. Diversos estudios han asociado factores genéticos y ambientales con el desarrollo de la EP. Las Glutation S Transferasa (GST) son una familia de enzimas que intervienen tanto en el metabolismo de toxinas como en la desintoxicación celular lo que hipotéticamente implica una función neuroprotectora. Las GST más ampliamente estudiadas en relación a la EP son la GSTM1 y la GSTT1. Aunque se ha sugerido una asociación entre mutaciones nulas en estos genes y la EP, también hay estudios que sugieren que no existe, por lo que se ha propuesto que dicha asociación dependería de la población analizada. Por tal motivo, el objetivo de este trabajo fue determinar si existe una asociación entre las mutaciones nulas en *GSTT1* y *GSTM1* y la EP en población mexicana. Se llevó a cabo un estudio de casos (75 pacientes con diagnóstico de EP) y testigos (75 individuos sin enfermedad neurodegenerativa) los cuales fueron pareados por edad y género. Se obtuvo DNA de sangre periférica y se realizó la genotipificación por PCR de punto final. Se realizaron las pruebas UPDRS, minimental y Hamilton para evaluar la severidad de la EP, estado cognitivo y depresión, respectivamente. La media de edad tanto para casos como para testigos fue de 70 años. Al comparar los resultados de las pruebas de minimental y Hamilton entre casos y testigos, solo la escala de Hamilton presentó diferencias estadísticamente significativas ($p<0.001$), siendo mayor en los casos que en los testigos. La media para los casos del UPDRS fue de 66.63. La mutación nula *GSTT1* se presentó en 7 de los casos y 11 de los testigos y la mutación nula en *GSTM1* en 27 de los casos y 26 de los testigos. Al comparar ambos grupos no encontramos diferencias estadísticamente significativas ni para la mutación nula en *GSTT1* ni para *GSTM1* ($p=0.314$ y $p=0.864$, respectivamente). En conclusión, los resultados sugieren que no existe asociación entre las mutaciones nulas en *GSTT1* y *GSTM1* y la EP.

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XLII Congreso Nacional de Genética Humana

MÉRIDA, YUCATÁN, Noviembre 29 a Diciembre 2 de 2017

La Asociación Mexicana de Genética Humana

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ASOCIACIÓN MEXICANA DE GENÉTICA HUMANA A.C.

CENTRO DE INVESTIGACIONES REGIONALES "DR. HIDEYO NOGUCHI" UNIVERSIDAD AUTÓNOMA DE YUCATÁN MÉXICO

Elizabeth I. Antuna-Salcido, Cosme Alvarado-Esquivel, Edna M. Méndez-Hernández, Jesús Hernández-Tinoco, Luís F. Sánchez-Anguiano, Francisco X. Castellanos-Juárez, Osmel La Llave-León, José M. Salas-Pacheco y Ada Sandoval-Carrillo

Por la presentación del trabajo libre en modalidad Cartel:

VARIANTES DEL GEN *TNF-α* Y SU ASOCIACIÓN CON DEPRESIÓN EN MUJERES EMBARAZADAS

Mérida, Yucatán, diciembre 1 de 2017

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Director CIR Dr. Hideyo Noguchi UADY

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UNIVERSIDAD JUÁREZ DEL ESTADO DE DURANGO
INSTITUTO DE INVESTIGACIÓN CIENTÍFICA

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A ***Elizabeth I. Antuna-Salcido, Cosme Alvarado-Esquivel, Edna M. Méndez-Hernández, Jesús Hernández-Tinoco, Luís F. Sánchez-Anguiano, Francisco X. Castellanos-Juárez, Osmel La Llave-León, José M. Salas-Pacheco y Ada Sandoval-Carrillo***

Por la presentación del trabajo ***"VARIANTES DEL GEN TNF-α Y SU ASOCIACIÓN CON DEPRESIÓN EN MUJERES EMBARAZADAS"****, realizado en las Jornadas Académicas "La Investigación Científica, Compromiso y Pertinencia Social", en el marco conmemorativo del XLVIII Aniversario del IIC y II Encuentro de Investigación de la DES - Ciencias de la Salud de la UJED.*

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A ***Cárdenas De la Cruz M. J., Ramos Rosales D.F., Barraza Salas M., Salas Pacheco J.M., Castellanos Juárez F.X., Olivas Linares O.L., Méndez Hernández E.M***

Por la presentación del trabajo ***"ANÁLISIS POSTMORTEM DE POLIMORFISMOS Y PERFILES DE EXPRESIÓN DE LOS GENES HMGCR, SREBP2, SOAT1 Y CYP46A1 Y SU ASOCIACIÓN CON SUICIDIO"****, realizado en las Jornadas Académicas "La Investigación Científica, Compromiso y Pertinencia Social", en el marco conmemorativo del XLVIII Aniversario del IIC y II Encuentro de Investigación de la DES - Ciencias de la Salud de la UJED.*

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CNG2018 006

ANÁLISIS POSTMORTEM DE POLIMORFISMOS Y PERFILES DE EXPRESIÓN DE LOS GENES *HMGCR*, *SREBP2*, *SOAT1* Y *CYP46A1* Y SU ASOCIACIÓN CON SUICIDIO

Cardenas De la cruz MJ[1], Ramos Rosales DF[2], Barrasa Salas M[2], Salas Pacheco JM[1], Olivas Linares OL[3], Mendez Hernandez EM[4]*

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Diversos estudios epidemiológicos han permitido asociar la hipocolesterolemia con un aumento del riesgo de suicidio, donde se ha sugerido que la concentración cerebral de colesterol desempeña un papel fundamental sobre los receptores de diversos neurotransmisores. El presente estudio pretende demostrar el papel que desempeñan diversos polimorfismos involucrados en la síntesis de colesterol como rs3761740 y rs3846662 del gen *HMGCR*, rs2228314 del gen *SERBP2*, rs1044925 del gen *SOAT1* y rs754203 del gen *CYP46A1* y la relación de éstos con el suicido. Estudio de casos y controles. Casos: sujetos cuya causa de fallecimiento sea suicidio. Controles: sujetos pareados por edad, sexo e intervalo post-mortem cuya causa de muerte indique un origen accidental no relacionado a padecimientos neurosiquiátricos. Se toma una muestra sanguínea de nivel periférico así como de tejido cerebral. Tamaño de la muestra: 150 en cada grupo. Se efectuó la genotipificación de los polimorfismos mencionados y perfiles de expresión de los genes *HMGCR, SERBP2*, *SOAT1* y *CYP46A1* en tejido cerebral y sangre periférica. Se cuantificaron los niveles de colesterol y 24S-hidroxicolesterol cerebral y los niveles de colesterol en suero. Para el presente análisis se reclutaron 142 sujetos. De estos, 79 casos y 63 controles, en el grupo de casos 63 (79.71%) hombres y 16 (20.36%) mujeres, con una media de edad de 32.73±14.98 años. Con respecto a los métodos de suicidio registrados, el 74.7% fue por obstrucción mecánica de vías aéreas, 19.0% intoxicación medicamentosa y 5.1% herida por arma de fuego. Sin embargo, no se observan diferencias al comparar las variables clínicas y los hábitos personales patológicos entre los sujetos con suicidio y controles. De manera preliminar, la variante rs2228314 del gen *SREBP2* se comporta como un factor protector asociado a suicidio en un modelo de herencia recesivo. No obstante, es importante contar con las mediciones de colesterol, 24S-hidroxicolesterol y expresión para correlacionar los resultados de la genotipificación con estos biomarcadores.

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JN-CAR-IC-56.

ASOCIACIÓN DE LOS POLIMORFISMOS RS206811, RS1042039, RS1054889 Y RS2073316 DEL GEN XDH CON LA PRESENCIA DE DETERIORO COGNITIVO EN EL ADULTO MAYOR

Ana Gabriela Fernández Chávez[1], Carolina Basio Salazar[2], Edna Madai Méndez Hernández[3], José Manuel Salas Pacheco[3], Abelardo Camacho Luis[4], Armando Ávila Rodríguez[4].
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INTRODUCCIÓN.

El deterioro cognitivo (DC) es un síndrome clínico caracterizado por la pérdida o el deterioro de las funciones mentales en distintos dominios conductuales y neuropsicológicos. Uno de los mecanismos propuestos para explicarlo es el estrés oxidativo. El ácido úrico (AU) es un poderoso antioxidante al cual se le atribuyen propiedades neuroprotectoras, su déficit ha sido asociado al desarrollo de enfermedades neurodegenerativas. Su síntesis está determinada por la enzima xantina deshidrogenasa (codificada por el gen XDH) por lo que alteraciones en los perfiles de expresión de este gen asociados a polimorfismos podrían incidir sobre una disminución en la síntesis de AU siendo este un factor de riesgo para el desarrollo de DC.

OBJETIVO.

Establecer si existe asociación entre los polimorfismos rs206811, rs1042039, rs1054889 y rs2073316 del gen XDH con la presencia de deterioro cognitivo en el adulto mayor.

METODOLOGÍA.

Estudio de casos y controles. Se incluyeron 164 adultos mayores de 60 años con presencia de DC y 164 sin la presencia de DC. La presencia de DC se avaluó con la escala MMSE. Se evaluaron los niveles séricos de AU y se realizó la genotifipicación de los polimorfismos rs206811, rs1042039, rs1054889 y rs2073316.

RESULTADOS.

Al comparar los niveles séricos de AU no se encontró diferencia significativa entre los grupos al comparar el nivel de deterioro o los genotipos. En cuanto a las frecuencias alélicas y genotípicas se encontró diferencia significativa (<0.05) en las frecuencias genotípicas de los polimorfismos rs1042039 y rs1054889 entre los grupos con y sin deterioro cognitivo.

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Contenido

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http://salud.durango.gob.mx/es/saludconciencia

CNG2018 025

CARACTERIZACION DE LAS VARIANTES rs1805386 del gen *LIG4* y rs1805377 del gen *XRCC4* y SU ASOCIACIÓN CON LA PREECLAMPSIA

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La preeclampsia (PE), enfermedad exclusiva del embarazo, es una de las principales causas de mortalidad materna en el mundo. Se caracteriza por presión arterial mayor de 140/90 mm/Hg y proteinuria mayor de 0,3 g/l después de las 20 semanas de gestación. Hoy en día se reconoce a la preeclampsia como un desorden placentario que tiene un origen genético multifactorial, es decir, es resultado de la interacción de genes y factores ambientales. A la fecha existen diversos estudios que demuestran que el daño al ADN es más elevado en pacientes con PE. Debido a esto, se ha propuesto que variantes en genes que participan en los procesos de reparación del ADN pueden asociarse a la PE. Por lo antes mencionado, el objetivo principal del presente trabajo fue determinar la asociación entre las variantes rs1805386 del gen *LIG4* y rs1805377 del gen *XRCC4* y la PE en mujeres de Durango. Se llevó a cabo un estudio transversal, observacional de casos y controles. Se incluyeron 155 mujeres con PE y 160 con embarazo normotenso. La genotipificación se realizó mediante PCR en tiempo real. Los controles presentaron una media de edad de 24.52±7.32 años y los casos de 23.53±6.8 años (p=0.083). Las medias de semanas de gestación fueron 37.95±3.54 y 35.38±5.30 para los controles y casos, respectivamente (p<0.001). El 30% de los controles y el 43.2% de los casos tuvo antecedentes de PE (p=0.015). Las frecuencias alélicas y genotípicas para la variante rs1805386 fueron T=0.90, C=0.10, T/T=0.84, T/C=0.11 y C/C=0.05 para los controles y T=0.93, C=0.07, T/T=0.85, T/C=0.14 y C/C=0.01 para los casos. Para la variante rs1805377 fueron G=0.62, A=0.38, G/G=0.37, G/A=0.49 y A/A=0.14 para los controles y G=0.6, A=0.4, G/G=0.4, G/A=0.4 y A/A=0.2 para los casos. No encontramos diferencias estadísticamente significativas para ninguna de las variantes al, comparar los grupos. Finalmente, se estimó la OR ajustando por edad y semanas de gestación; no encontramos asociación entre las variantes y la PE. En conclusión, nuestros resultados sugieren que en nuestra población, las variantes rs1805386 del gen *LIG4* y rs1805377 del gen *XRCC4* no se asocian con la PE.

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EL POLIMORFISMO *rs1800435* (*G177C)* DEL GEN *ALAD* COMO FACTOR DE RIESGO PARA INTOXICACIÓN POR PLOMO Y PREECLAMPSIA

La-Llave-León O[1]*, Salas-Pacheco JM[1], Salvador-Moysen J[1], García-Vargas G[2], Pérez-Morales R[3], Castellanos-Juárez FX[1], Sandoval-Carrillo A[1], Esquivel-Rodríguez E[4], Duarte-Sustaita J[2], Méndez-Hernández E[1]

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La exposición a tóxicos ambientales, como el plomo, se ha asociado con algunas complicaciones del embarazo como abortos y preeclampsia. Existe evidencia sobre la influencia de ciertos genes en la absorción y distribución del plomo en el organismo. Un gen implicado en la susceptibilidad a la toxicidad del plomo es *ALAD*, el cual codifica la ácido δ-aminolevulínico deshidratasa, una enzima que cataliza el segundo paso en la síntesis del grupo hemo en los eritrocitos. En algunas poblaciones se ha encontrado asociación entre el polimorfismo *rs1800435* (G177C) del gen *ALAD* y los niveles de plomo en sangre (NPS). Por su participación en mecanismos que desencadenan estrés oxidante, este polimorfismo podría estar involucrado también en los mecanismos explicativos de la fisiopatología de la preeclampsia; un síndrome que causa entre 70,000 y 80,000 muertes maternas cada año en el mundo. Para la posible asociación de este polimorfismo con los NPS y con la preeclampsia se realizó un estudio de casos y controles anidado en una cohorte de 462 mujeres embarazadas del estado de Durango. Durante el seguimiento, 63 mujeres sufrieron preeclampsia (casos) y fueron seleccionadas al azar 252 controles (cuatro por cada caso). Se determinó NPS por espectrofotometría de absorción atómica con horno de grafito y se realizó la genotipificación por PCR en tiempo real con sondas TaqMan. Las frecuencias genotípicas en la cohorte fueron de 0.92 para el homocigoto silvestre GG (ALAD1-1); 0.07 para el heterocigoto GC (ALAD1-2) y 0.01 para el homocigoto mutado CC (ALAD2-2). La prueba *t* de Student mostró NPS más altos en las portadoras del alelo polimórfico en comparación con el genotipo homocigoto silvestre (3.07 ± 5.20 µg/mL vs 1.94 ± 2.38 µg/mL; p = 0.037). Aunque el porcentaje de preeclampsia fue mayor entre las portadoras del alelo polimórfico (12.7% vs 6.3%), el análisis de regresión logística no mostró asociación entre el polimorfismo y la preeclampsia [OR = 2.15, IC 95% (0.87 – 5.27); p = 0.096)]. Los resultados sugieren la necesidad de realizar más investigación sobre la posible asociación entre este polimorfismo y el riesgo de sufrir preeclampsia.

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Por su participación como **Ponente** dentro de la

II Jornada Nacional de Investigación en Salud Durango 2018

Con el tema:

Niveles de Plomo en Sangre y su Asociación con la Actividad de la Enzima Ácido Delta-Aminolevulínico Deshidratasa

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los días 18, 19 y 20 de octubre del 2018, en el
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Por la presentación del trabajo ***"ASOCIACIÓN ENTRE LOS NIVELES DE PLOMO EN SANGRE Y LA ACTIVIDAD DE LA ENZIMA ÁCIDO DELTA-AMINOLEVULÍNICO DESHIDRATASA"****, realizado en las Jornadas Académicas "La Investigación Científica, Compromiso y Pertinencia Social", en el marco conmemorativo del XLVIII Aniversario del IIC y II Encuentro de Investigación de la DES - Ciencias de la Salud de la UJED.*

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Por la presentación del trabajo ***"RELACIÓN ENTRE LOS NIVELES DE PLOMO EN SANGRE Y LA EXPOSICIÓN OCUPACIONAL EN MUJERES EMBARAZADAS DE DURANGO"****, realizado en las Jornadas Académicas "La Investigación Científica, Compromiso y Pertinencia Social", en el marco conmemorativo del XLVIII Aniversario del IIC y II Encuentro de Investigación de la DES - Ciencias de la Salud de la UJED.*

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CERTIFICATE OF PARTICIPATION

It is certified, that:

Verónica Loera-Castañeda ; Wilma Espino-Vázquez; Jorge Díaz-García; Jorge Torres-Monreal; Susana Vázquez Simental; Ismael Lares-Asseff; IGNACIO VILLANUEVA-FIERRO; Diana Martínez-Montes; Marisela Bueno-Urbina; Guadalupe Godínez-Álvarez; Giovanni Rodríguez-Lozano; Luz Martínez-Díaz; José Salas-Pacheco; Irene Leal-Berúmen; Pilar Hernández-Rodríguez

Authors of the work:

MODIFICACIÓN DE MARCADORES DE RIESGO METABÓLICOS Y CARDIOVASCULARES EN PACIENTES CON SOBREPESO Y OBESIDAD POSTERIOR A 6 MESES DE TERAPIA COGNITIVO-CONDUCTUAL

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IBQ. Miguel Rosales Domínguez
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CENTRO DE INVESTIGACIONES REGIONALES "DR. HIDEYO NOGUCHI"
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MÉRIDA

Edith Maldonado-Soto, Sergio M. Salas-Pacheco, Ernesto G. Miranda-Morales, Edna M. Méndez-Hernández, Francisco X. Castellanos-Juárez, Osmel La Llave-León, Oscar Arias-Carrión, José M. Salas-Pacheco y Ada Sandoval-Carrillo

Por la presentación del trabajo libre en modalidad Cartel:

POLIMORFISMOS EN GENES DE REPARACION DE ADN Y SU ASOCIACIÓN CON LA ENFERMEDAD DE PARKINSON

Mérida, Yucatán, noviembre 30 de 2017

DR. JORGE E. ZAVALA CASTRO
Director CIR Dr. Hideyo Noguchi UADY

DRA. DORIS PINTO ESCALANTE
Presidente AMGH

DR. RODRIGO RUBI CASTELLANOS
Secretario AMGH

UNIVERSIDAD JUÁREZ DEL ESTADO DE DURANGO

INSTITUTO DE INVESTIGACIÓN CIENTÍFICA

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Constancia

A ***Edith Maldonado-Soto, Sergio M. Salas-Pacheco, Ernesto G. Miranda-Morales, Edna M. Méndez-Hernández, Francisco X. Castellanos-Juárez, Osmel La Llave-León, Oscar Arias-Carrión, José M. Salas-Pacheco y Ada Sandoval-Carrillo***

Por la presentación del trabajo ***"POLIMORFISMOS EN GENES DE REPARACION DE ADN Y SU ASOCIACIÓN CON LA ENFERMEDAD DE PARKINSON"****, realizado en las Jornadas Académicas "La Investigación Científica, Compromiso y Pertinencia Social", en el marco conmemorativo del XLVIII Aniversario del IIC y II Encuentro de Investigación de la DES - Ciencias de la Salud de la UJED.*

Atentamente

" Por mi raza hablará el espíritu "

Victoria de Durango, Dgo. a 05 de Octubre de 2018

Dr. Luis Francisco Sánchez Anguiano
Director del IIC

Dra. Laura Ernestina Barragán Ledesma
Representante de la DES
Ciencias de la Salud

COLEGIO MEXICANO DE INGENIEROS BIOQUÍMICOS, A.C.

CERTIFICATE OF PARTICIPATION

It is certified, that:

SUSUKY MAR ALDANA; EDNA MADAI MENDEZ HERNANDEZ; JOSE MANUEL SALAS PACHECO; MARCELO BARRAZA SALAS; OSCAR ARIAS CARRION

Authors of the work:

NIVELES DE VITAMINA D Y ÁCIDO ÚRICO Y SU RELACIÓN CON LA PRESENCIA DE ENFERMEDAD DE PARKINSON

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IBQ. Miguel Rosales Domínguez
President of the CMIBQ

Dra. Deifilia Ahuatzi Chacón
Chair Scientific Committee

JN-CAR-IC-14.

NIVELES DE VITAMINA D Y ÁCIDO ÚRICO Y SU RELACIÓN CON LA PRESENCIA DE ENFERMEDAD DE PARKINSON

Mar Aldana Susuky[1]; Salas Pacheco José Manuel[2]; Méndez Hernández Edna Madai[2,3].
1. Facultad de Medicina y Nutrición, Universidad Juárez del Estado de Durango.
2. Instituto de Investigación Científica, Universidad Juárez del Estado de Durango
3. Departamento de Investigación y Desarrollo Tecnológico, Servicios de Salud de Durango.

INTRODUCCIÓN.

La Enfermedad de Parkinson (EP) se presenta por deficiencia dopaminérgica secundaria a la degeneración de la sustancia nigra. Se han descrito niveles reducidos de ácido úrico (AU) y vitamina D (VitD) en pacientes con EP sin embargo, ninguno de estos estudios ha incluido a la población mexicana. Además poco se sabe acerca del papel de estos metabolitos en la fisiopatogenia de la enfermedad.

OBJETIVO.

Establecer si existe relación entre los niveles reducidos de VitD y AU con la presencia de EP.

MATERIAL Y MÉTODO.

Estudio de casos y controles. Definición de casos: Pacientes con EP. Definición de control: Sujetos sanos. Se evaluaron los niveles de AU, glucosa, perfil lipídico y VitD. Se aplicaron las escalas UPDRS (Escala Unificada para la Evaluación de la EP) y Hoehn y Yahr para avaluar la severidad de la EP en los casos.

RESULTADOS.

Se incluyeron 71 casos y 71 controles. Al comparar los niveles de AU entre los grupos de estudio se observa que los pacientes con EP muestran niveles más bajos de AU en comparación con el grupo control (4.5 $\pm$ 1.5 vs 5.0 $\pm$ 1.3, p 0.036). No se observaron diferencias estadísticamente significativas en los niveles de vitamina D (15.0 $\pm$ 5.8 vs 12.9 $\pm$ 7.0, p 0.095). Los pacientes con EP presentaron niveles significativamente menores de colesterol total en comparación con el grupo control (171.0 $\pm$ 44.1 vs 192.5 $\pm$ 51.1, p 0.011).

CONCLUSIONES.

En nuestra población, los pacientes con Enfermedad de Parkinson, presentaron niveles significativamente reducidos de ácido úrico y colesterol en comparación con el grupo control y no se observaron diferencias estadísticamente en los niveles de vitamina D. Los niveles de ácido úrico y vitamina D no se relacionaron con la severidad de la enfermedad de Parkinson, sin embargo, los niveles de colesterol total si se correlacionaron de manera inversa con la severidad de la enfermedad de Parkinson.

PALABRAS CLAVE.

Enfermedad de Parkinson, ácido úrico, vitamina D.

SALUD CON CIENCIA

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Otorga la presente:

Constancia

A ***Alfredo Martínez Juárez, José M. Salas Pacheco, Francisco X. Castellanos Juárez, Alma Rosa Pérez Álamos, Osmel La Llave León***

Por la presentación del trabajo ***"NIVELES DE PLOMO EN SANGRE Y MUERTE FETAL EN MUJERES EMBARAZADAS DE DURANGO"****, realizado en las Jornadas Académicas "La Investigación Científica, Compromiso y Pertinencia Social", en el marco conmemorativo del XLVIII Aniversario del IIC y II Encuentro de Investigación de la DES - Ciencias de la Salud de la UJED.*

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TRABAJO LIBRE - BDM00016

Prevalencia de anemia en un grupo de estudiantes de la Universidad Juárez Autónoma de Tabasco.

Méndez Bautista María Fernanda, Gordillo Jiménez Silvia Cristell, Flores Dorantes María Teresa, Pedraza Montero Pascual, Vilchis Reyes Miguel Ángel, Torres Sauret Quirino, Mendoza Lorenzo Patricia, Trejo Sánchez Blanca Estela

UNIVERSIDAD JUÁREZ AUTÓNOMA DE TABASCO

Introducción: En estado de anemia, los eritrocitos o glóbulos rojos resultan insuficientes para satisfacer las necesidades fisiológicas del organismo. Estas células transportan O2 y CO2 hacia los diferentes tejidos del cuerpo. La anemia se origina por la pérdida de sangre, la falta de producción de glóbulos rojos, las deficiencias nutricionales (hierro, ácido fólico, vitaminas B12, A, C), problemas hormonales, el embarazo, o bien, al aumento en la velocidad de destrucción de los glóbulos rojos. Estos antecedentes apoyan la idea de que la prevalencia de anemia podría ser considerada un indicador del estado de salud de una población.

Objetivo: Determinar la prevalencia de anemia en un grupo de estudiantes de la Universidad Juárez Autónoma de Tabasco (UJAT).

Metodología: Se realizó un estudio transversal retrospectivo que incluyo la revisión y compilación de 391 análisis clínicos de estudiantes de la División Académica de Ciencias Básicas de la UJAT realizados durante Febrero-Agosto del 2015. El análisis de los datos antropométricos (genero, edad, peso, talla, IMC) y de las Biometrías Hemáticas (hemoglobina, eritrocitos, HCT, VCM, HCM, CHCM) se realizaron en el programa IBM SPSS® 19.

Resultados: Dentro de la DACB, los estudiantes de las Licenciaturas de QFB y Geofísica fueron los grupos más representativos con el 48 y 42% del total de la población, mientras que Química y Física solo constituyeron el 7.4 y 2.6% respectivamente. El 53% de la población fueron mujeres, mientras que cerca del 47% fueron hombres, la edad promedio para ambos grupos fue de 19 años. El diagnóstico de anemia se realizó de acuerdo a los estándares establecidos por la OMS para hombres y mujeres no embarazadas. De acuerdo con este análisis, solo el 0.25% de la población estudiantil de la UJAT, presento Anemia de tipo Microcítica Hipocrómica. En este estudio no se identificó la presencia de Anemia Macrocítica.

Conclusiones: De acuerdo con la OMS, una prevalencia menor al 5% de anemia en las poblaciones es un indicativo de un estado de salud favorable, sin embargo resulta imperativo desarrollar estudios epidemiológicos de mayor alcance para incrementar nuestra calidad y certeza en el diagnóstico.

TRABAJO LIBRE - BDM00017

Factores de riesgo cardiovascular y metabólico asociados a prediabetes en niños en edad escolar

Mendez Hernandez Edna Madai[1], Escamilla Garcia Victor Manuel[2], Garcia Lara Liliana Guadalupe[3], Carrillo Leyva Pedro[3], Castellanos Juarez Francisco Xavier[1], Salas Pacheco José Manuel[1]

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Introducción: En 2003, la Asociación Americana de Diabetes reconoció la existencia de un grupo de sujetos cuyos niveles de glucosa resultan elevados para ser considerados normales pero no cumplen con los criterios para establecer el diagnóstico de diabetes, esta condición fue categorizada como Prediabetes. En población adulta, la presencia de prediabetes representa un factor de riesgo para el desarrollo de Diabetes y enfermedades cardiovasculares; sin embargo, poco se ha caracterizado esta problemática en niños.

Objetivo: Identificar los principales factores de riesgo cardiovascular y metabólico asociados al desarrollo de prediabetes en niños en edad escolar

Metodología: Estudio transversal analítico. Se estudiaron niños en edad escolar de instituciones públicas de educación básica de la ciudad de Durango. A todos los participantes se les realizaron las siguientes mediciones somatométricas: Peso, talla, índice de masa corporal, porcentaje de grasa corporal y tensión arterial. Se realizó toma de muestra sanguínea venosa para la cuantificación de los niveles de glucosa, perfil de lípidos, resistencia a la insulina y biometría hemática.

Resultados: Se incluyeron 489 niños, 249 hombres y 240 mujeres. Se identificaron 23 casos de niños con prediabetes (4.7%). La prevalencia de sobrepeso en el total de la muestra fue 17.2% (84 sujetos) mientras que la de obesidad fue de 12.5% (61 sujetos). El 23.7% (112 sujetos) presentaron hipertrigliceridemia y un 8.2% (40 sujetos) presentaron hipercolesterolemia. En el análisis de regresión logística la presencia de prediabetes se asoció significativamente con el porcentaje de grasa corporal (OR 1.056, IC95% 1.008-1.106), el nivel de triglicéridos (OR 1.013, IC95% 1.006-1.019) y colesterol (OR 1.015, IC95% 1.004-1.019). De igual forma, la presencia de hipertrigliceridemia (OR 4.297, IC95% 1.698-10.872) y resistencia a la insulina (OR 9.902, IC95% 4.094-23.952) se observaron fuertemente asociadas al desarrollo de prediabetes.

Conclusiones: La prevalencia de factores de riesgo cardiovascular y metabólico en la población escolar estudiada fue elevada. Las principales variables asociadas al desarrollo de prediabetes en niños fueron la presencia de hipertrigliceridemia y resistencia a la insulina, así como el porcentaje de grasa corporal y el nivel de colesterol.

REVISTA MEXICANA DE CIENCIAS FARMACÉUTICAS

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RESÚMENES
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En los últimos diez lustros han existido grande cambios que han revolucionado el mundo farmacéutico, ante los cuales es necesario una constante adaptación y reinvención. Así, es como la Asociación Farmacéutica Mexicana AC ha logrado trascender en el tiempo y cumplir medio siglo de ser la Asociación por Excelencia de los profesionales Farmacéuticos.

Ha sido una ardua labor cumplir con los altos estándares que se exigen en este sector, por lo que se ha requerido de un gran compromiso y competitividad para cumplir "50 años de Innovación, Experiencia y Excelencia".

Este año al celebrar la cuadragésima novena edición del Congreso Nacional de Ciencias Farmacéuticas y Séptima Edición Internacional, la AFMAC reafirma su contribución en la formación de profesionistas de calidad mundial, con un programa científico de alto nivel con prestigiados ponentes extranjeros y nacionales, expertos en sus respectivos campos de estudio, buscando que los contenidos sean de utilidad para acrecentar el conocimiento requerido en el ejercicio diario de las diferentes áreas que conforman el gremio farmacéutico. Por otra parte, con la presentación de los trabajos libres, una de las actividades fundamentales del congreso, se presentarán los esfuerzos de diferentes grupos de investigación; esto se logra con el intercambio de experiencias y opiniones de manera cordial y sencilla, sin perder objetividad y visualizar la actualidad de las Ciencias Farmacéuticas en México.
Finalmente, quiero aprovechar este espacio para hacer un afectuoso reconocimiento al trabajo realizado por el Comité Científico, el Consejo Directivo y el Personal de Apoyo de la Asociación Farmacéutica Mexicana AC, sin cuya participación no sería posible realizar esta reunión que nos permite trascender como gremio competitivo.

Dra. Elizabeth Sánchez-González
Directora de Ciencia y Tecnología
AFM AC

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TRABAJO LIBRE - BDM00024

Frecuencia de grupos sanguíneos en estudiantes de la Universidad Juárez Autónoma de Tabasco.

Custodio Adorno Karely Del Carmen, Gómez Frias Isela Natividad, Mendoza-Lorenzo Patricia, Flores Dorantes María Teresa, Pedraza-Montero Pascual, Trejo Sánchez Blanca Estela

UNIVERSIDAD JUÁREZ AUTÓNOMA DE TABASCO

Introducción: Los estudios sobre la frecuencia de los grupos sanguíneos ABO y Rh en la región sur-sureste del país son muy escasos. El estudio de estos antígenos y sus anticuerpos no solo son la base para las transfusiones sanguíneas y los transplantes de órganos en las diversas áreas de salud, sino que reportan otras importantes aplicaciones en el campo de la medicina y la investigación como su uso para la confirmación de pruebas de paternidad, la identificación de víctimas en medicina forense y estudios poblacionales.

Objetivo: Determinar la frecuencia de los diferentes grupos sanguíneos en estudiantes de cuatro Divisiones Académicas de la Universidad Juárez Autónoma de Tabasco (UJAT).

Metodología: Se realizó un estudio transversal descriptivo retrospectivo que incluyo la revisión y compilación de 3667 determinaciones de grupos sanguíneos de estudiantes de cuatro Divisiones Académicas (Ciencias Básicas, Ingeniería y Arquitectura, Sistemas Computacionales y la Multidisciplinaria Jalpa de Méndez) realizadas de Marzo del 2014 a Octubre del 2015. Con la edad, el género, la División Académica y la Licenciatura de cada uno de los participantes se genero una base de datos que fue analizada en el programa IBM SPSS® 19.

Resultados: El análisis de los datos mostró una mayor prevalencia del género masculino con respecto al género femenino, ambos con una edad promedio de 18 años. La División Académica con la mayor representatividad de participantes fue la División Académica de Ciencias Básicas, sobresaliendo sus Licenciaturas de Químico Farmacéutico Biólogo y Geofísica, seguida de la División Académica de Ingeniería y Arquitectura, con una importante representación de la Licenciatura de Ingeniería Civil. La División Académica que registro la menor participación fue la División Académica de Ingeniería y Sistemas. En lo que respecta a los grupos sanguíneos el grupo O+ reporto la mayor frecuencia, seguido de los grupos A+ y B+, mientras que los grupos menos representativos fueron el O-, el AB+, el A-, el B- y el AB-.

Conclusiones: La identificación de los grupos sanguíneos con mayor prevalencia en la región sur-sureste del país nos permitirá conocer y caracterizar mejor a nuestras poblaciones, sin olvidar el impacto positivo en la conservación de la salud de sus pobladores.

TRABAJO LIBRE - BDM00025

Hipocolesterolemia y su asociación con la presencia y severidad de la enfermedad de Parkison

Méndez Hernández Edna Madai[1], Ruano Calderón Luis Ángel[2], Quiñones Canales Gerardo[3], Vértiz Hernández Ángel Antonio[4], La Llave León Osmel[1], Arias Carrión Oscar[5], Salas Pacheco José Manuel[1]

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[5]HOSPITAL GENERAL "DR MANUEL GEA GONZALEZ" SS

Introducción: Recientemente diversos autores han sugerido que alteraciones en el metabolismo lipídico pueden estar relacionadas con un riesgo incrementado de desarrollar de enfermedades neurodegenerativas incluyendo la Enfermedad de Parkinson, sin embargo, otros autores no han logrado confirmar esta asociación y el tema continúa en controversia.

Objetivo: Establecer si existe asociación entre niveles reducidos de colesterol con la presencia y severidad de la Enfermedad de Parkinson.

Metodología: Estudio de casos y controles. Definición de casos: Pacientes con EP. Definición de control: Sujetos sanos. Se evaluaron los niveles de colesterol total y sus fracciones del perfil lipídico. Se aplicaron las escalas UPDRS (Escala Unificada para la Evaluación de la EP) y Hoehn y Yahr para avaluar la severidad de la EP en los casos.

Resultados: Se incluyeron 71 casos y 179 controles. Al realizar un análisis comparativo entre los grupos de estudio se observó que los pacientes con Enfermedad de Parkinson muestran niveles más bajos de colesterol en comparación con el grupo control (170.7 ± 51.9 vs 187.7 ± 52.4, p 0.024). Se realizó una comparación entre aquellos sujetos que presentaron diferentes grados de severidad de la enfermedad observando diferencias estadísticamente significativas entre las categorías leve, moderada y severa (186.7±45.6 vs 161.0±40.1 vs 140.1±27.4 respectivamente, p 0.006)

Conclusiones: Nuestros resultados son consistentes con estudios previos realizados en otras poblaciones en los que se reporta la presencia de niveles reducidos de colesterol en sujetos con Enfermedad de Parkinson. Al respecto, el colesterol es una biomolécula fundamental para la organización de las membranas neuronales y para la formación y mantenimiento de las "balsas lipídicas" (lipid rafts) implicadas en numerosos aspectos de la función cerebral, tales como señalización de factores de crecimiento y transmisión sináptica. Por esta razón, la deficiencia de colesterol cerebral podría generar importantes consecuencias. Este mecanismo podría explicar la relación observada entre niveles reducidos de colesterol con una mayor severidad de la enfermedad

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La Asociación Farmacéutica Mexicana, A.C.
otorga la presente

CONSTANCIA

A Edna Madai Mendez Hernandez, Victor Manuel Escamilla Garcia, Liliana Guadalupe Garcia Lara, Francisco Xavier Castellanos Juarez, José Manuel Salas Pacheco, Pedro Carrillo Leyva

Por la presentación del trabajo:

Factores de riesgo cardiovascular y metabólico asociados a prediabetes en niños en edad escolar

durante XLIX Congreso Nacional de Ciencias Farmacéuticas
y VII Congreso Internacional de Ciencias Farmacéuticas
Huatulco - 4 al 7 de septiembre 2016

QFB. Victoria Ruiseco
Presidencia AFM

Dra. Elizabeth Sánchez González
Dirección de Ciencia y Tecnología

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Trabajo 41

Variantes génicas y perfiles de expresión de genes reguladores de la síntesis de colesterol cerebral (HMGCR, SREBP2 y CYP46A1) y su asociación con la presencia de Trastorno Depresivo Mayor

Ponente: MENDEZ HERNANDEZ EDNA MADAI

(1)Méndez Hernández Edna Madai; (2) Marcela Araceli Segoviano Mendoza; (2) Manuel de Jesús Cárdenas de la Cruz; (3) Oscar Arias Carrión; (2) Marcelo Barraza Salas; (2) Francisco Xavier Castellanos Juarez; (2) José Manuel Salas Pacheco

(1)Hospital Regional De Alta Especialidad Ixtapaluca; (2) Universidad Juárez del Estado de Durango; (3) Hospital General "Dr. Manuel Gea González

Área: BIOMEDICA

Antecedentes. Evidencia reciente sugiere que las alteraciones en la biosíntesis del colesterol representan un mecanismo asociado con el desarrollo del trastorno depresivo mayor (TDM) al afectar las vías de señalización serotoninérgicas centrales. La enzima de unión al elemento regulador de esteroles 2 (SREBP-2), la enzima 3-hidroxi-3-metilglutaril-CoA reductasa (HMGCR) y la enzima colesterol 24-hidroxilasa (CYP46A1) participan en la síntesis del colesterol cerebral. Los polimorfismos en los genes SREBP-2, HMGCR y CYP46A1 y sus perfiles de expresión podrían estar asociados con TDM. Por lo tanto, investigamos este vínculo entre los polimorfismos y los perfiles de expresión de los genes HMGCR, SREBP-2 y CYP46A1 y MDD en 424 sujetos adultos de la población mexicana mestiza.

Objetivo. Establecer si existe asociación entre los polimorfismos y perfiles de expresión de los principales genes reguladores de la síntesis de colesterol cerebral (HMGCR, SREBP2 y CYP46A1) con la presencia de Trastorno Depresivo Mayor

Material y Métodos. Estudio de casos y controles que incluyó 212 sujetos deprimidos y 212 controles sanos. Los polimorfismos rs2228314, rs376174, rs3846662 y rs754203 y los perfiles de expresión se analizaron usando rtPCR. Se determinaron los niveles plasmáticos de 24 S-hidroxicolesterol, colesterol, triglicéridos y colesterol de lipoproteínas de alta densidad (HDL-c) y colesterol de lipoproteínas de baja densidad (LDL-c).

Resultados. La frecuencia de hipocolesterolemia fue significativamente mayor en sujetos con TDM (31.13% vs 13.68%, p <0.001). El genotipo heterocigoto de rs3846662 se asoció con MDD (OR 1.72 IC95% 1.076-2.761, p 0.02). Los niveles de expresión del gen SREBP-2 fueron significativamente más bajos (p <0.002), mientras que los niveles de expresión del gen HMGCR fueron significativamente más altos (p <0.023) en el grupo con TDM en comparación con el grupo control. Una correlación inversa entre los niveles de lípidos en suero y los niveles de expresión del gen HMGCR (r -0.331, p 0.005).

Conclusión. Nuestros resultados muestran por primera vez una asociación entre los polimorfismos HMGCR rs3846662 y MDD así como entre los perfiles de expresión del perfil de los genes SREBP-2 y HMGCR con MDD en la población mexicana mestiza

75 AÑOS
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XXIII ENCUENTRO NACIONAL DE INVESTIGADORES DE LA SECRETARÍA DE SALUD

San Juan del Río, Querétaro del 17 al 20 de octubre 2018

JN-CAR-IC-44.

PARAMETROS HEMATOLÓGICOS EN SUJETOS DURANGUENSES CON ENFERMEDAD DE PARKINSON

Miranda Morales Ernesto Gerardo[1], Castellanos Juárez Francisco Xavier[1], La Llave León Osmel[1], Méndez Hernández Edna Madai[1], Sandoval Carrillo Ada[1], Quiñones Canales Gerardo[2], Ruano Calderón Luis Ángel[3], Arias Carrión Oscar4 y Salas Pacheco José Manuel[1].

1. Instituto de Investigación Científica, UJED-Durango
2. Hospital General Santiago Ramón y Cajal-ISSSTE, Durango
3. Hospital General 450, Durango
4. Hospital General Dr. Manuel Gea González, Ciudad de México

INTRODUCCIÓN.

Los parámetros hematológicos y su asociación con la Enfermedad de Parkinson (EP) han sido poco descritos. En particular, el incremento en los niveles de Hemoglobina (Hb) se ha asociado con una mayor incidencia de EP.

OBJETIVO.

Determinar si existen diferencias en los parámetros hematológicos entre sujetos con EP y un grupo control.

MATERIALES Y MÉTODOS.

Estudio de 35 casos de EP y 35 controles en sujetos que acudieron al Hospital General 450, el Hospital Santiago Ramón y Cajal del ISSSTE y Ciudad del Anciano en Durango.

RESULTADOS.

Los niveles de Hb fueron de 14.71±2.02 dL y 14.21±2.02 dL, en casos y controles, respectivamente (p = 0.315). El recuento de glóbulos rojos (RBC) de casos fue de 4.72±0.51 y de los controles de 4.74±0.63 (p = 0.939). El volumen corpuscular medio (VCM) de 90.72±7.73 en casos y 91.62±4.98 en controles (p = 0.264). El Hematocrito (HCT) se encontró en 42.87±5.75 en casos y 43.35±5.54 en controles (p = 0.485). La Hemoglobina corpuscular media (HCM) fue de 31.08±2.11 en casos y 30.05±2.20 en controles (p = 0.049). Finalmente, la concentración de la hemoglobina corpuscular media (CHCM) se encontró en 34.35±2.04 en casos y 32.79±1.81 en controles (p < 0.001).

CONCLUSIÓN.

Aunque los niveles de Hb fueron ligeramente mayores en los casos, no se encontraron diferencias significativas. Sin embargo, si se observaron diferencias significativas en los niveles de HCM y CHCM. Futuros estudios con tamaños muestrales mayores son necesarios para corroborar estos hallazgos en nuestra población.

PALABRAS CLAVE.

Enfermedad de Parkinson, parámetros hematológicos, hemoglobina.

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JN-CAR-IC-45.

NIVELES DE ÁCIDO ÚRICO EN SUJETOS DE DURANGO CON ENFERMEDAD DE PARKINSON

Miranda Morales Ernesto Gerardo[1], Castellanos Juárez Francisco Xavier[1], La Llave León Osmel[1], Méndez Hernández Edna Madai[1], Sandoval Carrillo Ada[1], Quiñones Canales Gerardo[2], Ruano Calderón Luis Ángel[3], Arias Carrión Oscar[4], Salas Pacheco José Manuel[1].

1. Instituto de Investigación Científica, UJED.-Durango
2. Hospital General Santiago Ramón y Cajal-ISSSTE, Durango
3. Hospital General 450. Durango
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INTRODUCCIÓN.

La función del ácido úrico (AU) y su efecto fisiopatológico en casos de EP en México han sido poco descritos. El AU ejerce un efecto antioxidante en neuronas y se conoce como un quelador de hierro. Previos reportes en otras poblaciones han asociado niveles bajos de AU con la EP.

OBJETIVO.

Determinar si existen diferencias en los niveles séricos de AU entre casos de EP y controles en población Duranguense.

MATERIAL Y MÉTODO.

Estudio de 61 casos de EP y 69 controles en sujetos que acudieron al Hospital General 450, el Hospital Santiago Ramón y Cajal del ISSSTE y Ciudad del Anciano en Durango.

RESULTADOS.

Encontramos niveles de AU de 5.35 $\pm$ 2.30 mg/dL para los casos y 6.03 $\pm$ 1.31 mg/dL para los controles (p = 0.010). Al estratificar por género, los niéveles de AU en mujeres con EP fue de 5.35 $\pm$ 1.46 mg/dL y de 5.62 $\pm$ 1.24 mg/dL para el grupo control (p = 0.442). En el grupo de hombres los niveles de AU fueron de 5.52 $\pm$ 6.45 mg/dL para los casos con EP y 6.46 $\pm$ 1.26 mg/dL para los controles (p = 0.008).

CONCLUSIONES.

Existen diferencias estadísticamente significativas en los niveles de AU, siendo menores en los individuos con EP. Al estratificar por género, observamos que esta diferencia solamente se mantiene en los hombres. Nuestros resultados concuerdan con lo previamente reportado en otras poblaciones.

PALABRAS CLAVE.

Enfermedad de Parkinson, ácido úrico, antioxidante.

SALUD CON CIENCIA

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JN-CAR-IC-46.

CARACTERIZACIÓN DE LAS VARIANTES H1/H2 DE MAPT Y RS1801133 DE MTHFR EN SUJETOS MEXICANOS CON ENFERMEDAD DE PARKINSON

Miranda Morales Ernesto Gerardo[1], Castellanos Juárez Francisco Xavier[1], La Llave León Osmel[1], Méndez Hernández Edna Madai[1], Sandoval Carrillo Ada[1], Quiñones Canales Gerardo[2], Ruano Calderón Luis Ángel[3], Arias Carrión Oscar[4], Salas Pacheco José Manuel[1].

1. Instituto de Investigación Científica, UJED-Durango
2. Hospital General Santiago Ramón y Cajal-ISSSTE, Durango
3. Hospital General 450, Durango
4. Hospital General Dr. Manuel Gea González, Ciudad de México

INTRODUCCIÓN.

Se han identificado mutaciones y polimorfismos en genes relacionados con la Enfermedad de Parkinson (EP). No obstante, las bases genéticas, y bioquímicas asociadas a la EP han sido poco estudiadas en nuestro país.

OBJETIVO.

Genotipificar los haplotipos H1/H2 de MAPT y rs1801133 del gen MTHFR en sujetos con EP e individuos sanos. Posteriormente, se determinará si estos polimorfismos están asociados a cambios epigenéticos.

MATERIAL Y MÉTODO.

Estudio de 108 casos y 91 controles en sujetos que acudieron al Hospital General Dr. Manuel Gea González en la Ciudad de México, el Hospital General 450, el Hospital Santiago Ramón y Cajal del ISSSTE y Ciudad del Anciano en Durango.

RESULTADOS.

Las frecuencias para los genotipos H1/H2 de MAPT fueron H1/H1: 0.80, H1/H2: 0.18 y H2/H2: 0.02, con respecto a los casos y H1/H1: 0.85, H1/H2: 0.14, y H2/H2: 0.01, con respecto a los controles. Las frecuencias genotípicas para el SNP rs1801133 de MTHFR fueron C/C: 0.19, C/T: 0.47, y T/T: 0.34, con respecto a los casos y C/C: 0.23, C/T: 0.53, y T/T: 0.24, para los controles. No se observaron diferencias estadísticamente significativas al comparar las frecuencias alélicas y genotípicas entre los casos y los controles (p = 0.3600 para H1/H2 de MAPT y p = 0.1450 para rs1801133 de MTHFR). Al estratificar por región (centro y norte del país) tampoco se observaron diferencias.

CONCLUSIONES.

Nuestros resultados sugieren que las variantes estudiadas no se asocian con la EP en la población estudiada.

PALABRAS CLAVE.

Enfermedad de Parkinson, MAPT, MTHFR.

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UNIVERSIDAD JUÁREZ DEL ESTADO DE DURANGO
INSTITUTO DE INVESTIGACIÓN CIENTÍFICA

Otorga la presente:

Constancia

A ***Miranda Morales Ernesto Gerardo, Castellanos Juárez Francisco Xavier, La Llave León Osmel, Méndez Hernández Edna Madai, Sandoval Carrillo Ada, Quiñones Canales Gerardo, Ruano Calderón Luis Ángel, Arias Carrión Oscar y Salas Pacheco José Manuel***

Por la presentación del trabajo ***"PARÁMETROS HEMATOLÓGICOS EN SUJETOS DE DURANGO CON ENFERMEDAD DE PARKINSON"****, realizado en las Jornadas Académicas "La Investigación Científica, Compromiso y Pertinencia Social", en el marco conmemorativo del XLVIII Aniversario del IIC y II Encuentro de Investigación de la DES - Ciencias de la Salud de la UJED.*

Atentamente

" Por mi raza hablará el espíritu "

Victoria de Durango, Dgo. a 05 de Octubre de 2018

Dr. Luis Francisco Sánchez Anguiano
Director del IIC

Dra. Laura Ernestina Barragán Ledesma
Representante de la DES
Ciencias de la Salud

UNIVERSIDAD JUÁREZ DEL ESTADO DE DURANGO
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Constancia

A ***Miranda Morales Ernesto Gerardo, Castellanos Juárez Francisco Xavier, La Llave León Osmel, Méndez Hernández Edna Madai, Sandoval Carrillo Ada, Quiñones Canales Gerardo, Ruano Calderón Luis Ángel, Arias Carrión Oscar y Salas Pacheco José Manuel***

Por la presentación del trabajo ***"CARACTERIZACIÓN DE LAS VARIANTES H1/H2 DE MAPT y rs1801133 DE MTHFR EN SUJETOS MEXICANOS CON ENFERMEDAD DE PARKINSON"****, realizado en las Jornadas Académicas "La Investigación Científica, Compromiso y Pertinencia Social", en el marco conmemorativo del XLVIII Aniversario del IIC y II Encuentro de Investigación de la DES - Ciencias de la Salud de la UJED.*

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INSTITUTO DE INVESTIGACIÓN CIENTÍFICA

Otorga la presente:

Constancia

A ***Miranda Morales Ernesto Gerardo, Castellanos Juárez Francisco Xavier, La Llave León Osmel, Méndez Hernández Edna Madai, Sandoval Carrillo Ada, Quiñones Canales Gerardo, Ruano Calderón Luis Ángel, Arias Carrión Oscar y Salas Pacheco José Manuel***

Por la presentación del trabajo ***"NIVELES DE ÁCIDO ÚRICO EN SUJETOS DE DURANGO CON ENFERMEDAD DE PARKINSON"****, realizado en las Jornadas Académicas "La Investigación Científica, Compromiso y Pertinencia Social", en el marco conmemorativo del XLVIII Aniversario del IIC y II Encuentro de Investigación de la DES - Ciencias de la Salud de la UJED.*

Atentamente

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Victoria de Durango, Dgo. a 05 de Octubre de 2018

Dr. Luis Francisco Sánchez Anguiano
Director del IIC

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JN-CAR-IC-26.

GENOTIPIFICACIÓN DE LAS VARIANTES GÉNICAS rs737267, rs6449213, rs734553, rs733175 DEL GEN SLC2A9 Y SU RELACIÓN CON LA CONCENTRACIÓN SÉRICA DE ÁCIDO ÚRICO EN ADULTOS MAYORES

María Elisa Moran Chaidez[1], Edgar Daniel García Reyes[1], María Fernanda Rodríguez Villa[1], Elizabeth Burciaga Favela[1], Aidé Danyre Cervantes Balderrama[1], Dr. Luis Enrique Herrera Jiménez 1,3, M. en C. Carolina Basio Salazar[1], L.N. Pedro Carrillo Leyva[1], L.P. Ana Gabriela Fernández Chávez[1], Dr. en C. José Manuel Salas Pacheco[2], Dra. en C. Edna Madai Méndez Hernández[2].

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INTRODUCCIÓN.

SLC2A9 es el principal locus asociado a las concentraciones séricas de ácido úrico (S[AU]) codificando GLUT9. Han identificado polimorfismos asociados a variaciones de S[AU], no se conoce su efecto en población mexicana.

OBJETIVO.

Genotipificar las variantes génicas de SLC2A9 y evaluar la relación con S[AU] en adultos mayores.

MATERIAL Y MÉTODO.

Estudio transversal comparativo. Se incluyeron 272 adultos mayores. A partir de muestra sanguínea venosa se cuantificaron S[AU] y se realizó extracciones de ADN para su análisis en qPCR, genotipificando rs737267, rs6449213, rs734553, rs733175 de SLC2A9. Se describieron las frecuencias alélicas y genotípicas y se realizó un análisis de varianza para identificar diferencias significativas en las S[AU] entre los genotipos de cada variante.

RESULTADOS.

Al comparar las S[AU] entre los genotipos de la variable rs737267 se observaron diferencias significativas (p0.033) entre homocigotos mutados (4.0±1.1mg/dl) y homocigotos silvestre (4.9±1.7mg/dl). De igual manera, la presencia del genotipo homocigoto mutado de la variante rs734553 redujo de forma significativa las S(AU) al compararlas con el genotipo homocigoto silvestre (4.0±1.1 versus 4.9±1.8mg/dl, p0.031). Al comparar S[AU] entre los genotipos de las variantes rs6449213 y rs733175 no se observaron diferencias estadísticamente significativas (p 0.80 y 0.51 respectivamente).

CONCLUSIÓN.

Se observaron diferencias significativas de S[AU] entre los polimorfismos rs737267 y rs734553, con concentraciones más bajas, sobre todo en los homocigotos mutados. Lo observado coincide con poblaciones japonesas, chinos y croatas. rs733175 tiende a disminuir los niveles de AU, pero no alcanzó significancia estadística (p0.051). En la variantes rs6449213 no se observaron variaciones significativas entre genotipos.

PALABRAS CLAVE.

Concentraciones séricas de ácido úrico, mutaciones, glut9, slc2a9.

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JN-CAR-IC-30.

GENOTIPIFICACIÓN DE LAS VARIANTES GÉNICAS rs505802, rs893006, rs2021860, rs1529909 DEL GEN SLC22A12 Y SU RELACIÓN CON LA CONCENTRACIÓN SÉRICA DE ÁCIDO ÚRICO EN ADULTOS MAYORES

María Elisa Moran Chaidez[1], Edgar Daniel García Reyes[1], María Fernanda Rodríguez Villa[1], Elizabeth Burciaga Favela[1], Aidé Danyre Cervantes Balderrama[1], Luis Enrique Herrera Jiménez[1,3], Carolina Basio Salazar[1], Pedro Carrillo Leyva[1], Ana Gabriela Fernández Chávez[1], José Manuel Salas Pacheco[2], Edna Madai Méndez Hernández[2].

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INTRODUCCIÓN.

SLC22A12, codifica URAT1, importante modulador de las concentraciones séricas de ácido úrico (S[AU]). Se identificaron polimorfismos asociados a variaciones de las S[AU], no se ha caracterizado su efecto en población mexicana.

OBJETIVO.

Genotipificar estas variantes génicas en adultos mayores de la ciudad de Durango y evaluar su relación con las S[AU].

MATERIAL Y MÉTODO.

Estudio transversal comparativo. Incluyó 333 adultos mayores de Durango. Se cuantificaron S[AU], y realizaron extracciones de ADN para la genotificación rs505802, rs893006, rs2021860 y rs1529909 de SLC22A12 por qPCR. Se describieron las frecuencias alélicas y genotípicas; se realizó un análisis de varianza para identificar diferencias significativas S[AU] entre los genotipos de cada variante, posteriormente se analizó post hoc de Bonferroni.

RESULTADOS.

Al comparar S[AU] entre los genotipos de la variante rs505802 se observaron diferencias significativas (p0.01) entre homocigotos mutados (5.23±1.81mg/dl) y heterocigotos (4.48±1.65mg/dl). Al estratificar por género se observaron diferencias significativas de S[AU] sólo en el grupo de hombres (p0.00), específicamente entre genotipos homocigoto silvestre (5.4±1.4mg/dl) y homocigoto mutado (6.2±2.0mg/dl). Las variantes rs2021860, rs1529909 y rs893006 no tuvieron diferencias estadísticamente significativas. Al estratificar por género, la variante rs1529909 mostró diferencias significativas (p0.02) en mujeres, específicamente entre homocigoto silvestre (3.9±1.2mg/dl) y heterocigoto (4.77± 1.78mg/dl).

CONCLUSIÓN.

Los homocigotos mutado de rs505802 y heterocigotos de rs1529909 incrementaron significativamente S[AU] en hombres y mujeres respectivamente. A este respecto, el hallazgo observado en la variante rs505802 es consistente con lo reportado previamente en población china; lo observado con la variante rs1529909 contrasta con lo descrito en población coreana.

PALABRAS CLAVE.

Concentraciones séricas de ácido úrico, mutaciones, urat1, sc22a12

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ANF0010

"INNOVACIÓN Y DESARROLLO FARMACÉUTICO
CON IMPACTO EN LA SALUD Y BIENESTAR DE LA POBLACIÓN"

LI Congreso Nacional de CIENCIAS FARMACÉUTICAS

ASOCIACION FARMACEUTICA MEXICANA

La Asociación Farmacéutica Mexicana. A.C.
otorga el presente

RECONOCIMIENTO a:

Francisco Javier Moreno Cruz, Lidia Verónica Juárez Moreno, Maribel Cervantes Flores, Ángel Antonio Vértiz Hernandez, José Manuel Salas Pacheco, Eduardo Lozano Guzmán

Por la presentación del trabajo:

Desarrollo y validación de un método analítico por HPLC para determinación de ivermectina en plasma humano

Septiembre 2018, Puerto Vallarta, Jalisco.

Dr. Efrén Hernández Baltazar
Presidencia de la Asociación Farmacéutica Mexicana, A. C.

Dra. Verónica Rodríguez López
Dirección de Ciencia y Tecnología de la Asociación Farmacéutica Mexicana, A. C.

XLII Congreso Nacional de Genética Humana

MÉRIDA, YUCATÁN, Noviembre 29 a Diciembre 2 de 2017

La Asociación Mexicana de Genética Humana

otorga la presente Constancia a:

ASOCIACIÓN MEXICANA DE GENÉTICA HUMANA A.C.

CENTRO DE INVESTIGACIONES REGIONALES "DR. HIDEYO NOGUCHI"
UNIVERSIDAD AUTÓNOMA DE YUCATÁN
MÉXICO

Alma Cristina Salas Leal, Francisco Xavier Castellanos Juárez, Osmel La Llave León, Edna Madai Méndez Hernández, Gerardo Quiñones Canales, Luis Ángel Ruano Calderón, Oscar Arias Carrión, José Manuel Salas Pacheco y Ada Sandoval Carrillo

Por la presentación del trabajo libre en modalidad Cartel:

CARACTERIZACIÓN DE LOS PERFILES DE EXPRESIÓN DEL GEN *SNCA* Y SU VARIANTE rs356219 EN PACIENTES CON ENFERMEDAD DE PARKINSON

Mérida, Yucatán, diciembre 1 de 2017

DR. JORGE E. ZAVALA CASTRO
Director CIR Dr. Hideyo Noguchi UADY

DRA. DORIS PINTO ESCALANTE
Presidente AMGH

DR. RODRIGO RUBI CASTELLANOS
Secretario AMGH

JN-CAR-IC-47.

CARACTERIZACIÓN DE POLIMORFISMOS EN LOS GENES SNCA, UBE2K, ALDH1A1, HSPA8, SKP1A Y PSMC4 EN SUJETOS CON ENFERMEDAD DE PARKINSON

Salas Leal Alma Cristina[1], Castellanos Juárez Francisco Xavier[1], La Llave León Osmel[1], Méndez Hernández Edna Madai[1], Quiñones Canales Gerardo[2], Ruano Calderón Luis Ángel[3], Arias Carrión Oscar[4], Salas Pacheco José Manuel[1], Sandoval Carrillo Ada[1].

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2. Hospital General Santiago Ramón y Cajal-ISSSTE, Durango
3. Hospital General 450, Durango
4. Hospital General Dr. Manuel Gea González, Ciudad de México

INTRODUCCIÓN.

La enfermedad de Parkinson (EP) es una enfermedad neurodegenerativa que se manifiesta como una desregulación en el control del movimiento. La EP está caracterizada por la pérdida de dopamina y la presencia de cuerpos de Lewy, formados por ubiquitina y a-sinucleína. A la fecha se han realizado diversos estudios que han asociado variantes génicas con la EP en diversas poblaciones; sin embargo, este tipo de estudios son muy escasos en población mexicana.

OBJETIVO.

Determinar las frecuencias alélicas y genotípicas de los polimorfismos rs3764435 de ALDH1A1, rs234365 de PSMC4, rs2110585 de SKP1, rs305124 de UBE2K, rs2236659 de HSPA8 y rs356219 de SNCA y su asociación con la EP.

MATERIALES Y MÉTODOS.

Se reclutaron 45 casos y 70 controles. La genotipificación se realizó por PCR tiempo real. Los análisis se realizaron con el programa SNPStats.

RESULTADOS.

El análisis de las frecuencias alélicas y genotípicas evidenció que solo el polimorfismo rs356219 del gen SNCA es un factor de riesgo para la EP (OR=2.8, IC951.277-6.163, p=0.009). Las frecuencias alélicas para este polimorfismo fueron A=0.46, G=0.54 en controles y A=0.30, G=0.70, en casos. Las genotípicas fueron A/A=0.19, G/A=0.54, G/G=0.27 en controles y A/A=0.11, G/A=0.38, G/G=0.51 en casos.

CONCLUSIONES.

El alelo G del polimorfismo rs356219 del gen SNCA es más frecuente en los pacientes con EP. Nuestros resultados confirman lo reportado previamente en otras poblaciones en los que se ha observado que el alelo G incrementa el riesgo de la EP. No se observó asociación con ninguna de las otras variantes y la EP.

PALABRAS CLAVE.

Enfermedad de Parkinson, SNCA, rs356219.

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JN-CAR-IC-55.

CARACTERIZACIÓN DE LOS PERFILES DE EXPRESIÓN DEL GEN SNCA Y SU VARIANTE rs356219 EN PACIENTES CON ENFERMEDAD DE PARKINSON

Salas Leal Alma Cristina[1], Castellanos Juárez Francisco Xavier[1], La Llave León Osmel[1], Méndez Hernández Edna Madai[1], Quiñones Canales Gerardo[2], Ruano Calderón Luis Ángel[3], Arias Carrión Oscar[4], Salas Pacheco José Manuel[1], Sandoval Carrillo Ada[1].

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INTRODUCCIÓN.

La enfermedad de Parkinson (EP) es una enfermedad neurodegenerativa caracterizada por pérdida neuronal, disminución en la disponibilidad cerebral de dopamina y la presencia de cuerpos de Lewy. Estos están formados principalmente por la proteína a-sinucleína la cual es codificada por el gen SNCA. Los perfiles de expresión y variantes génicas han sido ampliamente estudiados en la EP en la búsqueda de posibles biomarcadores en sangre periférica.

OBJETIVO.

Determinar si el polimorfismo rs356219 modula los niveles de expresión del gen SNCA y si estos se asocian con la EP.

MATERIALES Y MÉTODOS.

Se reclutaron 15 casos y 15 controles pareados por edad y sexo. La cuantificación relativa de la expresión y la genotipificación se realizó por PCR tiempo real. Para el análisis estadístico se usaron pruebas para comparación de medias.

RESULTADOS.

Se obtuvieron expresiones similares del gen SNCA en individuos con EP con respecto a los controles sanos (0.751±0.32 vs 0.788±0.30; p=0.752). Los niveles de expresión en base al genotipo fueron 0.490±0.16 (A/A), 0.812±0.32 (A/G) y 0.72±0.17 (G/G). Al comparar en base a un modelo de herencia dominante, se observó una mayor expresión por la presencia de la variante alélica (A/A=0.490±0.16 vs A/G+G/G=0.788±0.29), con una tendencia a la significancia (p=0.057).

CONCLUSIONES.

No se observaron diferencias en los niveles de expresión entre los grupos lo cual concuerda con lo reportado por Tan y cols en 2005 en población china. Por otro lado, nuestros resultados sugieren que la presencia del alelo de riesgo se asocia con mayores niveles de expresión.

PALABRAS CLAVE.

Enfermedad de Parkinson, Perfiles de expresión, SNCA, rs356219

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ASOCIACIÓN DEL SNP RS3764435 DEL GEN ALDH1A1 CON ENFERMEDAD DE PARKINSON EN POBLACIÓN MEXICANA

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La Enfermedad de Parkinson (EP) es el segundo desorden neurodegenerativo más frecuente. Recientemente se han reportado nuevos descubrimientos acerca de factores genéticos implicados en esta enfermedad. El gen ALDH1A1 codifica para la enzima aldehído deshidrogenasa, involucrada en la degradación de productos neurotóxicos resultado del metabolismo de la dopamina. Se ha demostrado que los niveles de ALDH1A1 y su actividad, se encuentran disminuidos en pacientes con EP. Entre los polimorfismos de un solo nucleótido (SNP) que podrían modular los niveles de expresión, se encuentra el SNP rs3764435 (A/C). El objetivo principal de este estudio fue establecer si existe asociación entre el SNP rs3764435 del gen ALDH1A1y la EP. Se trata de un estudio de casos (119 pacientes con diagnóstico de EP) y controles (177 individuos sin enfermedad neurodegenerativa). Se obtuvo ADN de sangre periférica y se realizó la genotipificaión por PCR tiempo real. El grupo control presentó una frecuencia para el alelo A=0.47 y para el alelo C=0.53; las frecuencias genotípicas fueron A/A=0.24, A/C=0.47 y C/C=0.29. Con respecto a los casos, las frecuencias alélicas fueron A=0.57 y C=0.43 y las genotípicas A/A=0.27, A/C=0.60 y C/C=0.13. Encontramos diferencias estadísticamente significativas entre los grupos tanto en las frecuencias alélicas como en las genotípicas (p=0.022 y p=0.006, respectivamente). El análisis de la estimación de riesgo evidenció que el genotipo C/C del SNP rs356219 del gen ALDH1A1 es un factor protector tanto en un modelo de herencia codominante como en el recesivo (OR=0.38, IC95%=0.20-0.71 y OR=0.42, IC95%=0.20-0.86, respectivamente). Nuestros resultados sugieren que el genotipo C/C del SNP rs3764435 del gen ALDH1A1 es un factor de protección para la EP en población mexicana y debido a su posición intrónica, se sugiere que el SNP puede tener un efecto positivo en la actividad enzimática como resultado del splicing alternativo o incluso influir en el incremento de la expresión génica.

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Constancia

A ***Salas-Leal A C, Pérez-Gavilán Ceniceros J A, Salas-Pacheco J M, Arias-Carrión O, Quiñones-Canales G, Ruano-Calderón L A, Castellanos-Juárez F X, Méndez-Hernández E M, La Llave-León O, Sandoval-Carrillo A A.***

Por la presentación del trabajo ***"ASOCIACIÓN DEL SNP rs3764435 DEL GEN ALDH1A1 CON ENFERMEDAD DE PARKINSON EN POBLACIÓN MEXICANA"****, realizado en las Jornadas Académicas "La Investigación Científica, Compromiso y Pertinencia Social", en el marco conmemorativo del XLVIII Aniversario del IIC y II Encuentro de Investigación de la DES - Ciencias de la Salud de la UJED.*

Atentamente

" Por mi raza hablará el espíritu "

Victoria de Durango, Dgo. a 05 de Octubre de 2018

Dr. Luis Francisco Sánchez Anguiano
Director del IIC

Dra. Laura Ernestina Barragán Ledesma
Representante de la DES
Ciencias de la Salud

UNIVERSIDAD JUÁREZ DEL ESTADO DE DURANGO

INSTITUTO DE INVESTIGACIÓN CIENTÍFICA

Otorga la presente:

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Por la presentación del trabajo ***"α-SINUCLEINA Y ENFERMEDAD DE PARKINSON EN POBLACION MEXICANA",*** *realizado en las Jornadas Académicas "La Investigación Científica, Compromiso y Pertinencia Social", en el marco conmemorativo del XLVIII Aniversario del IIC y II Encuentro de Investigación de la DES - Ciencias de la Salud de la UJED.*

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Representante de la DES
Ciencias de la Salud

JN-CAR-IC-23.

PREVALENCIA DE LOS POLIMORFISMOS RS1805386 DEL GEN LIG4, RS2075686 Y RS6869366 DEL GEN XRCC4 INVOLUCRADOS EN MECANISMOS DE REPARACIÓN DE ADN EN POBLACIONES AUTÓCTONA Y MESTIZA

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INTRODUCCIÓN.

La integridad del material genético de las células está sometida continuamente a múltiples amenazas. En células eucariotas, es reparada principalmente mediante mecanismo de reparación por extremos no homólogos (NHEJ). En donde participan las proteínas XRCC4 y Ligasa IV de DNA, polimorfismos en dichas proteínas pueden producir aberraciones cromosómicas e inestabilidad genómica que pueden desembocar en el desarrollo de un proceso cancerígeno

OBJETIVO.

Determinar la frecuencia de los SNPs rs1805386 de LIG4, rs2075686 y rs6869366 de XRCC4 en las poblaciones autóctona y mestiza de la ciudad de Durango

MATERIAL Y MÉTODO.

Se realizó un estudio transversal analítico con dos grupos de estudio Grupo 1: Población autóctona de la ciudad de Durango, Dgo; Grupo 2: Población mestiza residente en la ciudad de Durango. Previa aprobación de los Comités de Ética e Investigación del Hospital General 450 de la Secretaria de Salud y de la Facultad de Medicina y Nutrición UJED se invitó a los pacientes que cumplieron los criterios de selección a participar en el estudio. Se realizó una historia clínica y toma de muestra sanguínea; para extracción de ADN y su posterior genotipificación mediante la técnica de PCR en tiempo real

RESULTADOS.

Se han incluido un total de 112 mujeres indígenas huicholas del municipio de Mezquital: Bancos de Calitique, 1 (0.89%); Los Fortiles, 26 (23.3%); Puerto de Guamúchil, 20 (17.8%); Huazamota, 11 (9.8%) Pilas, 47 (41.9%); Viboras, 1 (0.89%); Brasiles, 6 (5.3%). Media de edad de 37.6 $\pm$ 14.4 años

PALABRAS CLAVE.

XRCC4, LIG4, cáncer, mecanismos de reparación de ADN.

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TRABAJO LIBRE - BDM00008

Valores de referencia de hemoglobina glucosilada de una población entre 20 y 30 años

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Introducción: La diabetes mellitus tipo 2 (DM2) es una alteración metabólica, que se caracteriza por niveles elevados de glucosa, debido a una deficiencia en la producción o en la acción de la insulina; afecta a más de 11.5 millones de mexicanos en una edad entre 20 a 79 años. Es un padecimiento que favorece el desarrollo de complicaciones como retinopatías, nefropatías y enfermedades cardiovasculares. Existen criterios diagnósticos para la DM2, en donde a partir del 2010 la American Society of Diabetes (ADA) incorpora la hemoglobina glucosilada (HbA1c) como criterio de diagnóstico. La HbA1c es una proteína que sirve para estimar el promedio de glucosa de un paciente durante los últimos tres meses. Los valores de referencia, son magnitudes importantes que se utilizan para la interpretación médica de resultados clínicos y es fundamental que cada laboratorio de diagnóstico cuente con valores de referencia propios debido a que dependen de la etnia, la edad y el sexo.

Objetivo: Establecer valores de referencia de HbA1c en estudiantes de la Universidad Autónoma del Estado de Morelos (UAEM).

Metodología: Se realizó un estudio transversal con una muestra aleatoria de estudiantes de la UAEM. Los estudiantes interesados en participar firmaron una carta de consentimiento informado y contestaron un cuestionario socio-demográfico. Se realizarón mediciones antropométricas de peso, talla, circunferencia de cintura y presión arterial. Se tomó una muestra sanguínea para cuantificar los niveles de glucosa y de HbA1c. La determinación de HbA1c y glucosa se realizaron con técnicas inmunoenzimáticas con un equipo COBAS 111-Roche USA.

Resultados: Participaron 92 estudiantes entre 20 y 30 años de edad de las diferentes facultades la UAEM, obteniendo los siguientes resultados: 33% hombres y 59% mujeres, el valor promedio de glucosa en ayuno fue de 70.78 (±16.12) mg/dl, la media de la población de HbA1c fue de 5.41% (±0.232), y los intervalos de referencia obtenidos fueron: límite inferior de 4.94% y un límite superior de 5.88%

Conclusiones: Se obtuvo un intervalo de referencia para esta población de 4.94% a 5.88%, intervalo con una diferencia de 0.19%, pero cerca del valor reportado por la ADA y la OMS (< 5.7%) en personas sanas.

TRABAJO LIBRE - BDM00009

Genotipificación del polimorfismo rs2228001 del gen XPC en mujeres con preeclampsia y embarazo normoevolutivo

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Introducción: La preeclampsia es un síndrome clínico caracterizado por hipertensión con disfunción orgánica múltiple, proteinuria y edema. Las bases moleculares de los factores causales implicados en la patogenia de la preeclampsia no son muy claras. Genes asociados con la prevención o reparación del ADN se han propuesto como candidatos potenciales para ser estudiados y determinar su posible rol en el desarrollo de la preeclampsia. El gen *XPC* codifica para una proteína indispensable del sistema de reparación por escisión de nucleótidos. El polimorfismo rs2228001 (T/G) ha sido asociado a distintos tipos de cáncer incluyendo el de pulmón y vejiga, sin embargo, no hay estudios que evalúen su posible asociación con la preeclampsia.

Objetivo: Determinar si existe asociación entre el polimorfismo rs2228001 del gen *XPC* y la preeclampsia.

Metodología: Estudio prospectivo de casos (diagnóstico de preeclampsia) y controles (embarazo normoevolutivo). De sangre periférica se extrajo ADN utilizando el sistema QIAamp DNA Blood Mini Kit. La genotipificación se realizó utilizando sondas Taqman. Se usaron medidas de tendencia central y de dispersión para los datos descriptivos, para las diferencias entre grupos se usaron las pruebas T de Student y Chi cuadrada. Para la estimación del riesgo (Odds Ratio, OR) se utilizó el software SNPstats.

Resultados: Al comparar los grupos (100 casos y 194 controles) se encontraron diferencias estadísticamente significativas en la TA sistólica y diastólica, semanas de gestación e IMC. En la edad y antecedente de preeclampsia no hubo diferencias entre los grupos. Las frecuencias alélicas fueron T=0.65, G=0.35 (controles), T=0.72 y G=0.28 (casos). Las frecuencias genotípicas fueron T/T=0.42, T/G=0.47, G/G=0.11 (controles), T/T=0.53, T/G=0.39, G/G=0.08 (casos). Aunque estadísticamente no se encontró asociación entre el polimorfismo rs2228001 y la preeclampsia al estimar la OR en los modelos de herencia codomiante, dominante y recesivo, el modelo dominante muestra una tendencia hacia la probabilidad de que este polimorfismo sea un factor protector (OR=0.64, IC95=0.39-1.04).

Conclusiones: Aunque las evidencias sugieren que el polimorfismo rs2228001 del gen *XPC* no se relaciona con la preeclampsia, es necesario realizar nuevos estudios con tamaño de muestra mayores en población mexicana para confirmar nuestros resultados.

TRABAJO LIBRE - BDM00012

Frecuencia de SNPs del gen MDR1 en pacientes de cardiología del HRAEB tratados con digoxina

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Introducción: La glicoproteína P es producto del gen de resistencia a multi- fármacos (MDR1 o ABCB1) y sus variantes genéticas pueden influenciar la biodisponibilidad y farmacocinética de varios fármacos. Tres polimorfismos (SNPs), C3435T, G2677T/A y C1236T, se han asociado al desequilibrio en la función de PGP y su genotipificación podría resultar de gran importancia para el tratamiento farmacológico personalizado de ciertos pacientes. La frecuencia genotípica ayudaría a catalogar la importancia del diagnóstico molecular.

Objetivo: Evaluar la frecuencia y genotipificación de los SNPs C3435T, C1236T y G2677 A/T del gen MDR1 en pacientes de cardiología del Hospital Regional de Alta Especialidad Bajío tratados con digoxina.

Metodología: Se obtuvo DNA genómico de 28 muestras de sangre completa de pacientes del área de cardiología del HRAEB tratados con digoxina, previa firma del consentimiento informado. Las muestras de sangre fueron obtenidas con EDTA como anticoagulante y el DNA fue purificado con el sistema Wizard Genomic DNA Purification kit. Los SNPs (C3435T, C1236T y G2677T/A) fueron identificados por MS-PCR, empleando cebadores específicos (M3435T/C/Rev; M1236T/C/Rev; M2677T/C/rev). Una vez obtenido el genotipo se calculó la frecuencia genotípica de la población incluida.

Resultados: De los 28 individuos incluidos en la población se observaron solo dos de los tres posibles genotipos para el SNP C1236T, con una frecuencia de CC(0.32) y CT(0.68). Para el SNP G2677T/A fueron cuatro de los seis genotipos posibles con una frecuencia de GA(0.04), TT(0.11), GG(0.21), GT(0.64). Para C3435T, se observaron los tres genotipos posibles con una frecuencia de CC (0.11), CT(0.86) y TT(0.03).

Conclusiones: Los genotipos más frecuentes fueron los heterocigotos CT, CT y GT para el SNP 3435, 1236 y 2677 respectivamente. Reportes sugieren que los homocigotos AA y TT para el SNP 2677 y TT para 3435 presentan menor expresión de PGP intestinal y un incremento en niveles plasmáticos de digoxina administrada vía oral, lo cual sugiere que al menos tres individuos con estos genotipos en la población estudiada pudieran tener un riesgo de intoxicación. Sin embargo es necesario realizar estudios de farmacovigilancia con el objetivo de demostrar la susceptibilidad que pudieran tener los pacientes con este genotipo.

TRABAJO LIBRE - BDM00013

Polimorfismos en IL6 e IL10 como factor de riesgo para la infección por *Toxoplasma gondii*

Salas Pacheco José Manuel[1], Acosta Cuevas Cesia Kerit[1], Hernández Díaz Karina[1], Sánchez Anguiano Luís Francisco[1], Hernández Tinoco Jesus[1], Alvarado Esquivel Comse[2], Castellanos Juarez Francisco Xavier[1]

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Introducción: La toxoplasmosis es una de las zoonosis parasitarias más comunes ocasionada por el protozoario *Toxoplasma gondii*, el cual infecta a todas las especies animales de sangre caliente incluyendo al humano. La respuesta inmune desempeña un papel en la determinación de la evolución de la enfermedad y, posiblemente, en la respuesta a la terapia convencional. Evidencias recientes demuestran que los polimorfismos -819 T/C del gen *IL-10* y -174 G/C del gen *IL-6* se asocian con bajos niveles de expresión. En virtud del papel que las interleucinas juegan en el proceso de respuesta inmune en contra de *Toxoplasma gondii* , diversos grupos de investigación han evaluado el efecto de estos y otros polimorfismos en los genes que las codifican.

Objetivo: Determinar si los polimorfismos -819 T/C del gen *IL-10* y -174 G/C del gen *IL-6* son un factor de riesgo para la toxoplasmosis.

Metodología: Estudio transversal integrado por 252 individuos del municipio de San Dimas en el estado de Durango. Se tomaron muestras de sangre periférica a partir de las cuales se extrajo ADN utilizando el sistema QIAamp DNA Blood. La genotipificación se realizó utilizando sondas Taqman. La infección por toxoplasma se evaluó determinando la presencia de anticuerpos IgG e IgM anti-*Toxoplasma gondii* mediante un inmunoensayo enzimático.

Resultados: Tomando en consideración la positividad (casos, n=169) o negatividad (controles, n=83) se conformaron los grupos de análisis. El 50% de la población fueron mujeres (n=126). De la población estudiada el 97.64% manifestó tener contacto con animales (gatos, perros, aves domésticas y/o animales de granja). Al analizar las frecuencias alélicas y genotípicas de ambos polimorfismos encontramos diferencias estadísticamente significativas en las frecuencias alélicas del polimorfismo -819 T/C (p=0.041) al comparar los grupos. Al estimar el riesgo que confieren estos polimorfismos en un modelo de herencia dominante, encontramos los siguientes resultados: una OR=1.06 (IC_{95}=0.59-1.92) para C/G-C/C en el polimorfismo -174 G/C y una OR=1.95 (IC_{95}=1.13-3.36) para T/C-C/C en el polimorfismo -819 T/C.

Conclusiones: El polimorfismo -819 T/C del gen *IL-10* puede ser un factor de riesgo para la toxoplasmosis en la población estudiada.

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La Asociación Farmacéutica Mexicana, A.C.
otorga la presente

CONSTANCIA

A *José Manuel Salas Pacheco*, *Rosa Arlette Medina Simental, Lino Enrique Ramírez Sosa, Francisco Xavier Castellanos Juarez, Osmel La Llave Leon, Edna Madai Méndez Hernández, Ada Sandoval Carrillo*

Por la presentación del trabajo:

Genotipificación del polimorfismo rs2228001 del gen *XPC* en mujeres con preeclampsia y embarazo normoevolutivo

durante XLIX Congreso Nacional de Ciencias Farmacéuticas y VII Congreso Internacional de Ciencias Farmacéuticas Huatulco - 4 al 7 de septiembre 2016

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TRABAJO LIBRE - BDM00026

Caracterización del polimorfismo *MTHFR* C677T en pacientes con enfermedad de Parkinson

Sandoval Carrillo Ada[1], *Medina Simental Rosa Arlette* [1], *Ramírez Sosa Lino Enrique*[1], *Méndez Hernández Edna Madai*[1], *Salas Pacheco José Manuel*[1], *Miranda Morales Ernesto Gerardo*[1], *Arias Carrion Oscar*[2]

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[2]HOSPITAL GENERAL MANUEL GEA GONZALEZ

Introducción: La enfermedad de Parkinson (EP) es uno de los trastornos neurodegenerativos más frecuentes. Clínicamente se caracteriza por bradicinesia, rigidez y temblor. Los defectos en la función motora se deben a una degeneración progresiva de las neuronas dopaminérgicas de la sustancia nigra pars compacta. Las bases genéticas y bioquímicas asociadas a la EP en población Mexicana han sido poco estudiadas. Diversos estudios han arrojado resultados controversiales al evaluar la posible asociación entre el polimorfismo *MTHFR* C677T y la EP. En el 2013 Wu y cols. encontraron una asociación entre este polimorfismo y la EP en población caucásica y asiática Sin embargo, en el 2013 Liao y cols. encontraron que no existe una asociación entre este polimorfismo y la EP en población China.

Objetivo: Determinar si existe una asociación entre el polimorfismo *MTHFR* C677T y la EP en población Mexicana.

Metodología: Estudio prospectivo de casos (n=100) y controles (n=100). Se tomaron muestras de sangre periférica a partir de la cual se extrajo ADN utilizando el sistema QIAamp DNA Blood Mini Kit. La genotipificación se realizó utilizando sondas Taqman en un equipo STEP ONE de 48 pozos.

Resultados: Al analizar los grupos encontramos una edad media de 69.4 años. 56% de nuestra población fueron mujeres. En el grupo de casos se encontró que el 9% tiene antecedentes familiares de EP, 16% discinesias y 35% trastornos del sueño. Las frecuencias alélicas fueron T=0.56 y C=0.44 para los controles y T=0.55 y C=0.45 para los casos. Las frecuencias genotípicas fueron T/T=0.29, T/C=0.53 y C/C=0.18 para los casos y T/T=0.33, T/C=0.43 y C/C=0.24 para los controles. No hubo diferencias estadísticamente significativas entre los grupos. Al estimar el riesgo (OR) de EP conferido por el polimorfismo en los modelos de herencia codominante, dominante y recesivo, no se encontró ninguna significancia estadística. Al estimar la OR estratificando por genero tampoco se encontraron significancias estadísticas.

Conclusiones: Los resultados de éste análisis muestran que el polimorfismo *MTHFR* C677T no es un factor de riesgo para la Enfermedad de Parkinson en población mexicana, resultado semejante al reportado en población China.

TRABAJO LIBRE - BDM00027

Polimorfismos en genes codificantes de transportadores de urato asociados con deterioro cognitivo en adulto mayor

Carrillo Leyva Pedro[1], *Méndez Hernández Edna Madai*[2], *Miranda Morales Ernesto Gerardo*[2], *Fernández Chávez Ana Gabriela*[1], *Castellanos Juárez Francisco Xavier*[2], *Basio Salazar Carolina*[2]

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Introducción: Diversos estudios han establecido asociación entre niveles reducidos de ácido úrico (AU) y un incremento en el riesgo de desarrollar deterioro cognitivo. Los principales reguladores del AU son los transportadores GLUT-9 (SLC2A9) y URAT-1 (SLC22A12). La presencia de variantes génicas en estos, influyen sobre los niveles de AU al modular su excreción por vía renal, por lo que pudieran representar factores de riesgo para padecimientos neurodegenerativos.

Objetivo: Establecer si existe asociación entre polimorfismos en genes codificantes de transportadores de urato asociados con la presencia y severidad de deterioro cognitivo en el adulto mayor.

Metodología: Estudio de casos y controles. Se estudiaron adultos mayores evaluados con la escala Mini-mental test de Folstein (MMSE) para identificar la presencia de deterioro o normalidad cognitiva. Se evaluaron niveles séricos y urinarios de AU. La genotipificación se realizó por qPCR.

Resultados: Se incluyeron 121 sujetos (31 casos, 90 controles). Al comparar las variables de estudio se observó edad (74.8±7.0 vs 72.7±6.8, p=0.201), IMC (25.9 (23.7-27.5) vs 28.5 (25.1-31.2), p=0.104), MMSE (19.0 (8-21) vs 29.0 (27.7-30.0), p=0.000), AU (4.1±1.7 vs 4.8±1.6, p=0.056), FEAU (31 (21.0-345.8 vs 23.8 (7.0-43.9), p=0.313), frecuencia de hipouricemia (0 (0) vs 5 (17.2), p 0.000), entre casos y controles respectivamente. En el análisis por género únicamente se observaron diferencias en las variables AU (3.4±1.7 vs 4.7±1.7, p=0.008) y MMSE (18.0 (5.0-20.0) vs 29.0 (26.7-30.0), p=0.000) al comparar entre casos y controles en las mujeres. En los hombres solamente se encontró diferencia en la variable MMSE (19 (8-21) vs 29.0 (28.0-30.0), p=0.000). No se observaron diferencias al comparar las frecuencias alélicas (p 0.712) y genotípicas (p 0.936) de la variante rs733175. Al comparar los niveles de AU entre los diferentes genotipos de esta variante observamos (4.65±1.7, 4.8±1.7 y 4.2±1.7, p=0.407) en homocigotos silvestres, heterocigotos y homocigotos mutados respectivamente.

Conclusiones: Se observaron niveles reducidos de AU en hombres con deterioro cognitivo, este comportamiento no se observó en las mujeres. El AU es un poderoso captador de radicales libres al cual se le atribuyen propiedades neuroprotectoras. Este pudiera ser el mecanismo por el cual pudiera asociarse a la funcionalidad cognitiva.

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TRABAJO LIBRE - BDM00028

Polimorfismos en los genes IL-18, IL-12, TLR4 y TLR9 y depresión en mujeres embarazadas

Sandoval Carrillo Ada[1], Salas Pacheco Sergio Manuel[1], Barragán Ávila Efrén[1], Alvarado Esquivel Cosme[2], Hernández Tinoco Jesus[1], Antuna Salcido Irasema[1], Salas Pacheco José Manuel[1]

[1]INSTITUTO DE INVESTIGACIÓN CIENTÍFICA DE LA UJED
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Introducción: Alrededor del 10% de las mujeres embarazadas y el 13 de las mujeres que acaban de dar a luz experimentan un desorden mental, principalmente depresión. Actualmente existe evidencia que demuestra que la depresión está asociada con una respuesta inflamatoria crónica de bajo grado, activación inmune mediada por células y activación compensatoria del sistema reflejo anti-inflamatorio. Por tal motivo, el estudio de variantes en genes que participan en estos procesos es de interés para diversos grupos de investigación, incluyendo el nuestro.

Objetivo: Determinar si existe asociación entre los polimorfismos IL-18 -137C/G, IL-12 1188A/C, TLR4 896A/G y TLR2 1350T/C y la depresión en mujeres embarazadas.

Metodología: Estudio de casos (embarazadas con depresión, n=153) y controles (embarazadas sin depresión, n=177). La depresión se evaluó mediante la Escala de Depresión de Post-Parto de Edinburgh. Se tomaron muestras de sangre periférica a partir de la cual se extrajo ADN utilizando el sistema QIAamp DNA Blood Mini Kit. La genotipificación se realizó por PCR en tiempo real utilizando sondas Taqman en un equipo STEP ONE de 48 pozos.

Resultados: Al analizar las frecuencias alélicas y genotípicas de los polimorfismos de estudio, solo encontramos diferencias estadísticamente significativa entre los grupos en el polimorfismo IL-12 1188A/C. Las frecuencias alélicas fueron A=0.64 y C=0.36 para los casos y A=0.55 y C=0.45 para los controles (p= 0.021). Las frecuencias genotípicas fueron A/A=0.39, A/C=0.51 y C/C=0.10 para los casos y A/A=0.31, A/C=0.49 y C/C=0.20 para los controles (p=0.038). De la misma forma, al estimar el riesgo mediante la OR, el genotipo C/C mostró ser un factor protector (OR=0.42, IC95=0.20-0.85). El alelo A se ha asociado con un incremento del 50% de transcritos por lo que nuestros resultados sugieren que aquellos individuos que presentan el genotipo C/C tendrían menores niveles de la interleucina 12 proinflamatoria. Como consecuencia, una respuesta inflamatoria disminuida estaría actuando como un factor protector para el desarrollo de la depresión.

Conclusiones: El genotipo C/C del polimorfismo A/C 1188 del gen IL-12 es un factor protector para la depresión en mujeres embarazadas.

TRABAJO LIBRE - BDM00030

Caracterización de los polimorfismos -857C/T y -238G/A del gen *TNF*α en mujeres embarazadas con depresión

Sandoval Carrillo Ada[1], Alvarado Esquivel Cosme[2], Sánchez Anguiano Luís Francisco[1], Hernández Tinoco Jesus[1], Salas Pacheco Sergio Manuel[1], Barragán Ávila Efrén[1], Salas Pacheco José Manuel[1]

[1]INSTITUTO DE INVESTIGACIÓN CIENTÍFICA DE LA UJED
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Introducción: La depresión es común, especialmente en mujeres con edad fértil, de las cuales entre un 8 a un 12% padecen de depresión. Se ha documentado que diferentes tipos de depresión mantienen una relación cercana con la respuesta inmunitaria, específicamente la inflamación y todas sus moléculas involucradas. *TNFα* tiene propiedades reguladoras de inflamación, mismas que juegan un papel crucial en la inmunidad innata y adaptativa. Polimorfismos en el gen *TNFα* se han asociado con variabilidad en su funcionalidad.

Objetivo: Determinar el papel de los polimorfismos -857C/T y -238G/A del gen *TNFα* en mujeres embarazadas con depresión.

Metodología: Estudio de casos (embarazadas con depresión, n=153) y controles (embarazadas sin depresión, n=177). La depresión se evaluó mediante la Escala de Depresión de Post-Parto de Edinburgh. De sangre periférica se extrajo ADN (QIAamp DNA Blood Mini Kit). Se genotipificó por PCR en tiempo real en un equipo STEP ONE de 48 pozos.

Resultados: La media de edad fue de 23.49 y 23.58 años (casos y controles, respectivamente). Las frecuencias alélicas y genotípicas del polimorfismo -857C/T fueron C=0.77, T=0.23, C/C=0.59, C/T=0.37, T/T=0.04 y C=0.84, T=0.16, C/C=0.72, C/T=0.25, T/T=0.03 para los casos y controles, respectivamente. Al comparar las frecuencias entre los grupos encontramos diferencias estadísticamente significativas en las alélicas (p=0.30) y genotípicas (p=0.047). Para el polimorfismo -238G/A las frecuencias alélicas y genotípicas fueron G=0.97, A=0.03, G/G=0.95, G/A=0.05, A/A=0 y G=0.92, A=0.08, G/G=0.84, G/A=0.16, A/A=0.2 para los casos y controles, respectivamente. Al comparar las frecuencias entre los grupos encontramos diferencias estadísticamente significativas en las alélicas (p=0.0019). Posteriormente se calcularon las OR encontrándose que el genotipo C/T del polimorfismo -857C/T es un factor de riesgo (OR=1.78, IC95=1.09-2.89), mientras que el genotipo G/A es un factor protector (OR=0.33, IC95=0.14-0.75). *TNFα* es un mediador de la respuesta inflamatoria en ambas direcciones, tanto proinflamatoria como antiinflamatoria, lo que quizá explique nuestros resultados. Estudios de funcionalidad para ambos polimorfismos son necesarios para determinar el papel que juega cada uno de ellos.

Conclusiones: El polimorfismo -857C/T del gen *TNFα* es un factor de riesgo y el polimorfismo -238G/A del gen *TNFα* es un factor protector, ambos, para la depresión en mujeres embarazadas.

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TRABAJO LIBRE - BDM00028

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Objetivo: Determinar si existe asociación entre los polimorfismos IL-18 -137C/G, IL-12 1188A/C, TLR4 896A/G y TLR2 1350T/C y la depresión en mujeres embarazadas.

Metodología: Estudio de casos (embarazadas con depresión, n=153) y controles (embarazadas sin depresión, n=177). La depresión se evaluó mediante la Escala de Depresión de Post-Parto de Edinburgh. Se tomaron muestras de sangre periférica a partir de la cual se extrajo ADN utilizando el sistema QIAamp DNA Blood Mini Kit. La genotipificación se realizó por PCR en tiempo real utilizando sondas Taqman en un equipo STEP ONE de 48 pozos.

Resultados: Al analizar las frecuencias alélicas y genotípicas de los polimorfismos de estudio, solo encontramos diferencias estadísticamente significativa entre los grupos en el polimorfismo IL-12 1188A/C. Las frecuencias alélicas fueron A=0.64 y C=0.36 para los casos y A=0.55 y C=0.45 para los controles (p= 0.021). Las frecuencias genotípicas fueron A/A=0.39, A/C=0.51 y C/C=0.10 para los casos y A/A=0.31, A/C=0.49 y C/C=0.20 para los controles (p=0.038). De la misma forma, al estimar el riesgo mediante la OR, el genotipo C/C mostró ser un factor protector (OR=0.42, IC95=0.20-0.85). El alelo A se ha asociado con un incremento del 50% de transcritos por lo que nuestros resultados sugieren que aquellos individuos que presentan el genotipo C/C tendrían menores niveles de la interleucina 12 proinflamatoria. Como consecuencia, una respuesta inflamatoria disminuida estaría actuando como un factor protector para el desarrollo de la depresión.

Conclusiones: El genotipo C/C del polimorfismo A/C 1188 del gen IL-12 es un factor protector para la depresión en mujeres embarazadas.

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Caracterización de los polimorfismos -857C/T y -238G/A del gen *TNF*α en mujeres embarazadas con depresión

Sandoval Carrillo Ada[1], *Alvarado Esquivel Cosme*[2], *Sánchez Anguiano Luís Francisco*[1], *Hernández Tinoco Jesus*[1], *Salas Pacheco Sergio Manuel*[1], *Barragán Ávila Efrén*[1], *Salas Pacheco José Manuel*[1]

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Introducción: La depresión es común, especialmente en mujeres con edad fértil, de las cuales entre un 8 a un 12% padecen de depresión. Se ha documentado que diferentes tipos de depresión mantienen una relación cercana con la respuesta inmunitaria, específicamente la inflamación y todas sus moléculas involucradas. *TNFα* tiene propiedades reguladoras de inflamación, mismas que juegan un papel crucial en la inmunidad innata y adaptativa. Polimorfismos en el gen *TNFα* se han asociado con variabilidad en su funcionalidad.

Objetivo: Determinar el papel de los polimorfismos -857C/T y -238G/A del gen *TNFα* en mujeres embarazadas con depresión.

Metodología: Estudio de casos (embarazadas con depresión, n=153) y controles (embarazadas sin depresión, n=177). La depresión se evaluó mediante la Escala de Depresión de Post-Parto de Edinburgh. De sangre periférica se extrajo ADN (QIAamp DNA Blood Mini Kit). Se genotipificó por PCR en tiempo real en un equipo STEP ONE de 48 pozos.

Resultados: La media de edad fue de 23.49 y 23.58 años (casos y controles, respectivamente). Las frecuencias alélicas y genotípicas del polimorfismo -857C/T fueron C=0.77, T=0.23, C/C=0.59, C/T=0.37, T/T=0.04 y C=0.84, T=0.16, C/C=0.72, C/T=0.25, T/T=0.03 para los casos y controles, respectivamente. Al comparar las frecuencias entre los grupos encontramos diferencias estadísticamente significativas en las alélicas (p=0.30) y genotípicas (p=0.047). Para el polimorfismo -238G/A las frecuencias alélicas y genotípicas fueron G=0.97, A=0.03, G/G=0.95, G/A=0.05, A/A=0 y G=0.92, A=0.08, G/G=0.84, G/A=0.16, A/A=0.2 para los casos y controles, respectivamente. Al comparar las frecuencias entre los grupos encontramos diferencias estadísticamente significativas en las alélicas (p=0.0019). Posteriormente se calcularon las OR encontrándose que el genotipo C/T del polimorfismo -857C/T es un factor de riesgo (OR=1.78, IC95=1.09-2.89), mientras que el genotipo G/A es un factor protector (OR=0.33, IC95=0.14-0.75). *TNFα* es un mediador de la respuesta inflamatoria en ambas direcciones, tanto proinflamatoria como antiinflamatoria, lo que quizá explique nuestros resultados. Estudios de funcionalidad para ambos polimorfismos son necesarios para determinar el papel que juega cada uno de ellos.

Conclusiones: El polimorfismo -857C/T del gen *TNFα* es un factor de riesgo y el polimorfismo -238G/A del gen *TNFα* es un factor protector, ambos, para la depresión en mujeres embarazadas.

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Por su participación como **Ponente en la Modalidad de Póster** dentro de la

II Jornada Nacional de Investigación en Salud Durango 2018

Con el tema:

Epidemiología de la Infección por Leptospira en la Población General de la Ciudad de Durango, México

los días 18, 19 y 20 de octubre del 2018, en el Centro Cultural y de Convenciones Bicentenario, Durango, Dgo.

Victoria de Durango, Dgo. octubre de 2018

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PERFILES DE EXPRESIÓN DE LOS GENES *HMGCR* Y *SREBP2* Y SU ASOCIACIÓN CON LA PRESENCIA DE TRASTORNO DEPRESIVO MAYOR

Segoviano Mendoza MA[1], Barraza Salas M[2], Castellanos Juárez FX[3], Salas Pacheco JM[3], Arias Carrión O[4], Méndez Hernández EM*[5]

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Se ha sugerido que la presencia de niveles reducidos de colesterol se asocia al desarrollo de Trastorno Depresivo (TD). En la síntesis de colesterol a nivel periférico y central, la proteína de unión a elementos reguladores de esteroles (SREBP2) y la enzima 3-hidroxi-3metil glutaril CoA reductasa (HMGCR) representan importantes blancos reguladores, por lo que sus niveles de expresión podrían estar asociados a la presencia de TD. Se incluyeron 35 casos de TD y 35 controles sanos. Se cuantificaron los niveles de colesterol en sangre venosa utilizando el método colorimétrico. Para el análisis de expresión génica, se utilizaron los kits *MagMax ambion* y *High Capacity cDNA reverse transcription* para la extracción y retrotranscripción. La cuantificación de la expresión relativa se realizó por qPCR. El análisis comparativo de los niveles de expresión del gen *SREBP2* reporta una expresión significativamente menor (p 0.025) en el grupo TD; la expresión de *HMGCR* tiene un incremento significativo en los casos comparada con los controles (p 0.0005). Se observó una correlación inversa entre los niveles de colesterol sérico y los niveles de expresión del gen *HMGCR (r -0.296, p 0.013)*. Se observó un patrón diferencial en los perfiles de expresión génica de *HMGCR* y *SREBP2* entre los grupos de estudio. Así mismo, la correlación inversa entre los niveles de colesterol y la expresión de *HMGCR* puede ser resultado de una adecuada respuesta del mecanismo de regulación transcripcional.

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Otorga la presente

CONSTANCIA a:

Jorge Alejandro Sosa Gutiérrez

Quien asistió y presentó el trabajo:

***Moringa oleifera* leaf extract preserves mitochondrial respiratory activity in HepG2 cells exposed to hyperglycemia**

Por:
Jorge Alejandro Sosa Gutiérrez, Mónica Valdez Solana, Maurizzio Battino, Alfredo Téllez Valencia, Claudia Domínguez Avitia, Gonzalo García Vargas, Oscar Flores Herrera, José Salas Pacheco, Erick Sierra Campos

En la modalidad de presentación oral durante el XXXI Congreso Nacional de Bioquímica del 6 al 11 de noviembre de 2016 en Aguascalientes, Ags.

Atentamente
Por el Comité Organizador

Dr. Miguel Lara Flores
Presidente

UNIVERSIDAD JUÁREZ DEL ESTADO DE DURANGO
INSTITUTO DE INVESTIGACIÓN CIENTÍFICA

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Constancia

A ***Ana Karem Sosa Hernández, Edna Madaí Méndez Hernández, Oscar Árias Carrión, José Manuel Salas Pacheco, Ada Agustina Sandoval Carrillo, Francisco Xavier Castellanos Juárez, Marcelo Barraza Salas***

Por la presentación del trabajo ***"CARACTERIZACIÓN DE LOS PATRONES DE EXPRESIÓN DE LOS GENES CLOCK Y VARIABLES POLISOMNOGRÁFICAS EN PACIENTES CON ENFERMEDAD DE PARKINSON"****, realizado en las Jornadas Académicas "La Investigación Científica, Compromiso y Pertinencia Social", en el marco conmemorativo del XLVIII Aniversario del IIC y II Encuentro de Investigación de la DES - Ciencias de la Salud de la UJED.*

Atentamente

" Por mi raza hablará el espíritu "

Victoria de Durango, Dgo. a 05 de Octubre de 2018

Dr. Luis Francisco Sánchez Anguiano
Director del IIC

Dra. Laura Ernestina Barragán Ledesma
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Ciencias de la Salud

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Rubella Immune Status in Pregnant Women in a Northern Mexican City

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Abstract

Background: The seroepidemiology of rubella virus infection in pregnant women in northern Mexico is largely unknown. We sought to determine the seroprevalence of rubella virus infection in pregnant women in the northern Mexican city of Durango, Mexico. Seroprevalence association with the socio-demographic, clinical and behavioral characteristics of the pregnant women was also investigated.

Methods: Through a cross-sectional study, we determined the seroprevalence of IgG and IgM anti-rubella virus in 279 pregnant women (mean age 29.17 ± 5.96 years; range 15 - 43 years) attending in a clinic of family medicine using enzyme-linked fluorescent assays. A questionnaire was used to obtain the socio-demographic, clinical and behavioral characteristics of the pregnant women. The association of rubella seropositivity and characteristics of the women was assessed by bivariate and multivariate analyses.

Results: Anti-rubella IgG antibodies (≥ 15 IU/mL) were found in 271 (97.1%) of the 279 pregnant women examined. None of the 279 pregnant women were positive for anti-rubella IgM antibodies. Multivariate analysis of socio-demographic, clinical and behavioral variables showed that seroreactivity to rubella virus was positively associated with national trips (OR = 7.39; 95% CI: 1.41 - 38.78; P = 0.01), and negatively associated with age (OR = 0.26; 95% CI: 0.06 - 0.99; P = 0.04).

Conclusions: Rate of rubella immunity in pregnant women in the northern Mexican city of Durango is high. However, nearly 3% of pregnant women are susceptible to rubella in our setting. Risk factors associated with rubella seropositivity found in this study may be useful for optimal design of preventive measures against rubella and its sequelae.

Keywords: Rubella; Pregnant women; Epidemiology; Seroprevalence; Cross-sectional study; Mexico

Introduction

Rubella virus is a single-stranded ribonucleic acid virus of the Togaviridae family [1, 2], and is a sole member of the genus Rubivirus [3]. Infection with rubella virus occurs by inhalation of contaminated droplets [1], and can be vertically transmitted to fetuses during maternal infection leading to congenital infection [4]. Rubella virus is an important pathogen worldwide [5]. Infection with rubella virus causes a febrile rash illness in children and adults [6]. In addition, infection with rubella virus in adults may cause severe inflammation and pain in the joints [1]. However, infection with rubella virus during the first trimester of pregnancy can lead to prematurity, low birth weight [7], miscarriage, stillbirth [6], and congenital rubella syndrome [6, 8]. This syndrome is characterized by fetal anomalies including mental retardation [9], heart defects, cataracts [8], blindness, deafness [9], and hepatomegaly and jaundice [10]. There is not currently antiviral treatment for rubella [1]. An effective and sure vaccine against rubella is available [1, 5]. However, rubella outbreaks in Japan and other countries have been reported recently [2, 5, 11].

The seroepidemiology of rubella virus infection in Mexican populations has been scantily studied. An 87% seroprevalence of anti-rubella antibodies in puerperal women from Delicias City in the northern Mexican city of Chihuahua was reported [12], whereas a 92.6% seroprevalence of rubella virus infection in pregnant women in two zones of the valley of Mexico was found [13]. In a study in Leon, Guanajuato, Mexico, researchers found a 71% seroprevalence of rubella in 176 women at reproductive age [14]. To the best of our knowledge, there is not any study on the seroepidemiology of rubella virus infection in pregnant women in northern Mexico. Therefore, this study was aimed to determine the seroprevalence of rubella virus infection in pregnant women in the northern Mexican city of Durango, Mexico. Furthermore, rubella seroprevalence

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association with the socio-demographic, clinical and behavioral characteristics of the pregnant women was also assessed.

Materials and Methods

Study design and population

We performed a cross-sectional study using stored serum samples from a previous survey of cytomegalovirus infection in pregnant women in Durango City, Mexico [15]. Samples were originally obtained to determine the seroprevalence of anti-cytomegalovirus antibodies in pregnant women attending a public primary health care center (Clinic of Family Medicine, Institute of Security and Social Services of State Workers) in Durango City, Mexico. Serum samples were obtained from April to November 2013. Inclusion criteria for enrollment of the participants were: 1) pregnant women attending prenatal care; 2) aged 15 years and older; 3) residence in Durango City; and 4) who voluntarily accepted to participate in the survey.

Socio-demographic, clinical and behavioral characteristics of participants

We obtained the socio-demographic, clinical and behavioral characteristics from the participants with the aid of a questionnaire. Socio-demographic items included age, birthplace, residence, educational level, occupation and socio-economic status. Clinical items included health status, history of lymphadenopathy, frequent headaches; impairments of memory, vision and hearing; and history of blood transfusions. In addition, the obstetric history (month of pregnancy, number of pregnancies, deliveries, cesarean sections and miscarriages) from each participant was recorded. Behavioral items included foreign traveling, alcohol consumption, tobacco use, and washing hands before eating.

Laboratory tests

Sera of the participants were kept frozen until analyzed. Sera were examined for anti-rubella IgG antibodies by a commercially available enzyme-linked fluorescent assay (ELFA) "VIDAS RUB IgG II" kit (bioMerieux SA, Marcy-l'Etoile, France) and for anti-rubella IgM antibodies by a commercially ELFA "VIDAS RUB IgM" kit (bioMerieux SA, Marcy-l'Etoile, France). Anti-rubella IgG antibody levels of $\geq$ 15 IU/mL were considered as a cut-off for seropositivity. This titer suggests protection against rubella [16, 17]. All tests were performed according to the manufacturer's instructions.

Ethical aspects

This study was performed using stored serum samples from a previous survey. In such previous study, the purpose and procedures of the study were explained to all participants, and a written informed consent was obtained from all of them and from the next of kin of minor participants. The ethical committee of the Institute of Security and Social Services of State Workers in Durango City, Mexico approved this study.

Statistical analysis

Results were analyzed with the aid of the Epi Info version 7 and SPSS version 15.0 software. For calculation of the sample size, we used a value of 15,000 as a population size from which the sample was selected, a reference seroprevalence of 87.0% [12] as expected frequency of the factor under study, 5.0% of confidence limits, a design effect of 1.0, one cluster, and a confidence level of 95%. The result of the calculation was 172 subjects. We evaluated the association between the characteristics of the women and rubella seropositivity by using bivariate and multivariate analyses. For comparison of the frequencies among groups, the Pearson's Chi-square and the Fisher exact test (when values were less than 5) were used. As a strategy to include variables in the multivariate analysis, we selected only variables with a P value equal to or less than 0.05 obtained in the bivariate analysis. We calculated the odds ratios (ORs) and 95% confidence intervals (CIs) by multivariate analysis using the Enter method. Statistical significance was set at a P value < 0.05.

Results

We enrolled a total of 279 pregnant women. Their mean age was 29.17 ± 5.96 years (range 15 - 43 years). Table 1 shows the general socio-demographic characteristics of the pregnant women studied. Anti-rubella IgG antibodies were found in 271 (97.1%) of the 279 pregnant women examined. None of the 279 pregnant women were positive for anti-rubella IgM antibodies. Of the socio-demographic characteristics of the pregnant women, the variables including age group and socioeconomic status were associated with anti-rubella IgG antibodies by bivariate analysis, whereas the variables including birthplace, residence, educational level, and occupation did not associate with anti-rubella IgG antibodies.

With respect to clinical characteristics, rubella seroprevalence was similar in ill and healthy pregnant women. Table 2 shows a correlation of rubella seroprevalence and clinical characteristics of pregnant women. Rubella seroprevalence was significantly (P = 0.02) higher in pregnant women with frequent headaches, whereas women with history of deliveries had a higher (borderline significance: P = 0.05) rubella seroprevalence than women without this history. Other clinical characteristics of women including history of lymphadenopathy, impairments of memory, vision and hearing; history of blood transfusions, month of pregnancy, number of pregnancies, deliveries, cesarean sections and miscarriages did not show an association with rubella seroprevalence by bivariate analysis.

Concerning behavioral characteristics of women, the variable "national trips" showed a borderline (P = 0.05) association with rubella seroprevalence. Other behavioral characteristics

Table 1. Socio-Demographic Characteristics of Pregnant Women and Seroprevalence of Rubella IgG Antibodies (≥ 15 IU/mL)

Characteristic	No. of women tested[a]	Rubella seroprevalence		P value
		No.	%	
Age groups (years)				
15 - 24	61	60	98.4	0.01
25 - 34	159	157	98.7	
35 - 43	59	54	91.5	
Birth place				
Durango State	259	252	97.3	1.00
Other Mexican State	16	16	100.0	
Residence place				
Durango State	278	270	97.1	1.00
Other Mexican State	1	1	100.0	
Residence area				
Urban	264	257	97.3	0.42
Suburban	4	4	100.0	
Rural	11	10	90.9	
Educational level				
Up to 6 years	1	1	100.0	0.30
7 - 12 years	102	97	95.1	
13 or more years	176	173	98.3	
Occupation				
Unemployed[b]	81	78	96.3	0.69
Employed[c]	198	193	97.5	
Socioeconomic level				
Low	15	13	86.7	0.02
Medium	257	252	98.1	
High	3	3	100.0	

[a]Sums may not add up to 279 because of some missing values. [b]Unemployed: none occupation, student or housewife. [c]Employed: employee, professional, business, or other.

including traveling abroad, alcohol consumption, tobacco use, and washing hands before eating did not show an association with rubella seroprevalence.

Multivariate analysis of socio-demographic, clinical and behavioral variables with P values ≤ 0.05 by bivariate analysis including age, socioeconomic status, frequent headache, number of deliveries, and national trips showed that seroreactivity to rubella was positively associated only with national trips (OR = 7.39; 95% CI: 1.41 - 38.78; P = 0.01), and negatively associated only with age (OR = 0.26; 95% CI: 0.06 - 0.99; P = 0.04).

Discussion

Very little is known about the serological status against rubella virus in pregnant women in Mexico. Therefore, this study aimed to determine the seroprevalence of IgG and IgM antibodies against rubella virus in pregnant women in the northern Mexican city of Durango. Results indicate that 97.1% of the pregnant women studied had protective (≥ 15 IU/mL) antibodies against rubella virus infection. In Mexico, vaccination against rubella started in 1998 [18]. Although the majority of pregnant women tested had protective antibodies, nearly 3% of women were susceptible to rubella. This figure seems low but considering that there are nearly 40,000 births a year in Durango State (http://cuentame.inegi.org.mx/monografias/informacion/dur/poblacion/dinamica.aspx?tema=me&e=10), thus there are about 1,200 pregnant women susceptible to rubella virus just in this Mexican state. Concerning studies in Mexico, the seroprevalence of rubella found in the present study is higher than the 87% seroprevalence of rubella in early puerperium women in the northern Mexican city of Delicias, Chihuahua [12], the 92.6% seroprevalence of rubella in pregnant women from Iztapalapa and Nezahualcoyotl areas in the valley of Mexico [13], and the 71% seroprevalence in women

Table 2. Bivariate Analysis of Clinical Data and Seropositivity to Rubella Virus in Pregnant Women in Durango City, Mexico

Characteristic	No. of women tested[a]	Rubella prevalence		P value
		No.	%	
Clinical status				
Healthy	267	260	97.4	0.27
Ill	11	10	90.9	
Lymphadenopathy ever				
Yes	42	41	97.6	1.00
No	237	230	97.0	
Headache frequently				
Yes	112	112	100.0	0.02
No	167	159	95.2	
Memory impairment				
Yes	63	63	100.0	0.20
No	216	208	96.3	
Hearing impairment				
Yes	20	20	100.0	1.00
No	259	251	96.9	
Visual impairment				
Yes	79	76	96.2	0.69
No	199	194	97.5	
Blood transfusion				
Yes	13	12	92.3	0.32
No	265	258	97.4	
Pregnancies				
One	89	89	100.0	0.11
Two	97	93	95.9	
Three	50	49	98.0	
Four	31	28	90.3	
Five	9	9	100.0	
More than 5	2	2	100.0	
Deliveries				
Zero	157	153	97.5	0.05
One	65	63	96.9	
Two	41	41	100.0	
Three	11	9	81.8	
Four	3	3	100.0	
More than 4	1	1	100.0	
Cesarean sections				
Zero	195	189	96.9	0.72
One	62	61	98.4	
Two	21	20	95.2	
Miscarriages				
Zero	223	217	97.3	0.88
One	46	44	95.7	
Two	8	8	100.0	
Three	1	1	100.0	
Month of pregnancy				
1 - 3	100.0	98	98.0	0.1
4 - 6	118	116	98.3	
7 - 9	56	52	92.9	

[a]Sums may not add up to 279 because of some missing values.

of reproductive age in Leon, Guanajuato [14]. However, this comparison should be taken with care since these studies were performed in different years and laboratory tests used were different from the tests we used. Previous seroprevalence studies in Mexico were performed from 1993 to 2004. In those years, the coverage of rubella vaccination was lower than the one in the recent years. We used ELFA to detect IgG antibodies against rubella virus, whereas in the previous studies, the hemagglutination inhibition method [13, 14] was used. In addition, we studied pregnant women in the urban city of Durango, whereas rural and urban women were enrolled in the study in Delicias, Chihuahua [12]. In an international context, the seroprevalence of rubella in pregnant women in Durango is higher than the 93.1% seroprevalence of rubella found in pregnant women seen in a tertiary hospital in Zaria, Nigeria [19], and 87.5% seroprevalence in pregnant women in Osogbo, Nigeria [20] using enzyme-linked immunosorbent assays. Similarly, our prevalence is higher than the 85.8% seroprevalence reported in pregnant women in southern Italy using a microparticle enzyme immunoassay [21]. The rubella seroprevalence found in our study is comparable with the 95.1% seroprevalence of rubella reported in pregnant women in Sudan [22], the 93.3% seroprevalence in pregnant women in Portugal [23], the 94.4% seroprevalence in pregnant women in Oslo, Norway [24], and 95.4% seroprevalence in women of childbearing age in Venezuelan Yupka indigenous communities [25].

We searched for factors associated with rubella seroprevalence. We found that seroreactivity to rubella was positively associated with national trips and negatively associated with age. International travel has been linked to rubella importation in the USA [26]. We did not find an association of international travel with rubella seropositivity. However, it is possible that rubella exposure occurs also by national trips as results of the present study suggests. Therefore, traveling to high endemic rubella regions should be avoided by pregnant women. In the present study, seroprevalence decreases with age. This fact might reflect the higher coverage of rubella vaccination in young women.

This study has limitations including a small sample size, and enrollment of women in only one clinic of family medicine. Further studies with larger sample sizes and in several clinics to determine the seroprevalence of rubella in Mexican communities should be conducted.

Conclusions

Rate of rubella immunity in pregnant women in the northern Mexican city of Durango is high. However, nearly 3% of pregnant women are susceptible to rubella in our setting. Risk factors associated with rubella seropositivity found in this study may be useful for optimal design of preventive measures against rubella and its sequelae.

Conflicts of Interest

The authors declare that no conflicts of interest exist.

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High Prevalence of *Toxoplasma gondii* Infection in Miners: A Case-Control Study in Rural Durango, Mexico

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Abstract

Background: Very little is known about the seroepidemiology of infection with the parasite *Toxoplasma gondii (T. gondii)* in miners. We determine the association of *T. gondii* infection and the occupation of miner, and the association of seropositivity for *T. gondii* with the socio-demographic, clinical, work and behavioral characteristics of the miners.

Methods: Through a case-control study, 125 miners working in Durango State, Mexico and 250 age- and gender-matched non-miner subjects were examined for the presence of anti-*T. gondii* IgG and IgM antibodies using enzyme-linked immunoassays. In addition, the presence of *T. gondii* DNA in miners was determined using polymerase chain reaction. Bivariate and multivariate analyses were used to determine the association of socio-demographic, work, clinical and behavioral characteristics of miners with *T. gondii* infection.

Results: Anti-*T. gondii* IgG antibodies were detected in 75 (60.0%) of 125 miners and in 55 (22.0%) of 250 controls (odds ratio (OR) = 5.31; 95% confidence interval (CI): 3.33 - 8.47; P < 0.001). Among IgG seropositive subjects, the frequency of anti-*T. gondii* IgM antibodies was significantly higher in miners (39/75, 52%) than in controls (8/55, 14.5%) (P < 0.001). All *T. gondii* seropositive miners referred themselves as healthy. Multivariate analysis of socio-demographic, housing, and behavioral characteristics of miners showed that *T. gondii* seropositivity was positively associated with being born in Durango State (OR = 3.44; 95% CI: 1.09 - 10.7; P = 0.03), consumption of boar meat (OR = 5.53; 95% CI: 1.49 - 20.3; P = 0.01), living in an overcrowded home (OR = 5.83; 95% CI: 1.49 - 22.8; P = 0.01), and was negatively associated with cleaning cat excrement (OR = 0.33; 95% CI: 0.11 - 0.90; P = 0.03) and consuming goat meat (OR = 0.16; 95% CI: 0.03 - 0.76; P = 0.02).

Conclusions: Surprisingly, our results indicate that miners represent a risk group for *T. gondii* infection. This is the first age- and gender-matched case-control study on the association of *T. gondii* infection and the occupation of miner. Further studies to identify the exact cause of high seropositivity in miners in rural Durango are needed.

Keywords: *Toxoplasma gondii*; Infection; Seroprevalence; Miners; Case-control study; Epidemiology; Mexico

Introduction

Infections with the parasite *Toxoplasma gondii (T. gondii)* are common in humans around the world [1]. These infections may lead to toxoplasmosis characterized by lymph node enlargement, chorioretinitis, or neuropsychiatric manifestations [2, 3]. Immunocompromised subjects infected with *T. gondii* may develop severe to life-threatening symptoms, most often toxoplasmic encephalitis [4]. In addition, a primary infection with *T. gondii* in pregnant women may lead to fetal infection and congenital toxoplasmosis [2, 5]. Infection with *T. gondii* is typically acquired by ingestion of raw or undercooked meat containing viable tissue cysts [6], or by ingestion of food or water contaminated with oocysts shed by cats [7]. Other routes of *T. gondii* infection are thought to be rare including organ transplantation [8] and blood transfusion [9].

The epidemiology of *T. gondii* infection in miners has been scantly studied, and we are not aware of any study of this infection in miners in Mexico. The epidemiological link between miners and *T. gondii* may be the close contact with soil and water that could be contaminated with oocyst shed by cats or other felids. In addition, miners work in rural areas where hunting of wild animals is common, and the risk for acquiring infection by eating raw or undercooked meat from *T. gondii*-infected animals is high. The seroprevalence of infection with *T. gondii* and its

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association with risk factors for infection in miners in Mexico are largely unknown. Therefore, we sought to determine the seroprevalence of *T. gondii* infection in miners in a municipality in rural Durango, Mexico, and to determine the association of seropositivity for *T. gondii* with the socio-demographic, clinical, work and behavioral characteristics of the miners.

Materials and Methods

Study design and population groups studied

We performed a case-control seroprevalence study of 125 miners (cases) and 250 age- and gender-matched non-miner subjects (controls). Cases and controls were examined for the presence of anti-*T. gondii* IgG and IgM antibodies. Miners were enrolled from December 2015 to August 2016 in a mine located in the San Dimas Municipality, in the northern Mexican state of Durango. Inclusion criteria for the miners were as follows: 1) working in the mine for at least 3 months, 2) 18 years and older, and 3) willing to participate in the study. All cases included in the study were males and had been working from 3 months to 47 years (mean: 11.4 ± 9.5 years) as miners. They were 20 - 87 (mean: 43.8 ± 14.6) years old. Controls were subjects randomly selected from the general population in rural Durango [10]. Controls were matched with cases by gender and age. Controls were males aged 20 - 87 (mean: 43.85 ± 14.5) years old and their age did not differ from that in cases (P = 0.99).

Socio-demographic, housing, clinical, work, and behavioral data of miners

Socio-demographic, clinical, work, and behavioral characteristics of the miners were recorded with the aid of a questionnaire. Socio-demographic items included age, gender, birthplace, residence, and socioeconomic level. Housing conditions items included availability of potable water, form of elimination of excretes, years of education of the head of the family, type of flooring at home, and crowding. Assessment of crowding was performed by dividing the number of people by the number of bedrooms in a house. "Semi-crowed" was considered when 1.6 - 3.5 people were living in a single bedroom. "Overcrowded" was considered when 3.6 or more people were living in a single room. The socioeconomic level of participants was self-reported. A "low socioeconomic status" was considered when a participant lived in poverty, whereas a "medium socioeconomic status" was considered when a participant did not live in poverty or wealth but in an intermediate status.

Clinical data included presence of any illness, history of lymphadenopathy, surgery, transplant, or blood transfusion, impairments in memory, reflexes, hearing and vision, frequent abdominal pain or headache, and dizziness. We recorded the duration of the activity as a miner for each participant. Behavioral items were contact with cats or other animals, cleaning cat feces, foreign traveling, type of meat consumed, frequency of meat consumption, eating raw or undercooked meat, animal brains, beef liver, and dried or cured meat, untreated water or unpasteurized milk, and unwashed raw vegetables or fruits. In addition, behavioral items included washing hands before eating, frequency of eating out of home (in restaurants or fast food outlets), alcoholism, tobacco smoking, and soil contact.

Detection of *T. gondii* IgG and IgM antibodies

Serum samples of miners were analyzed for anti-*T. gondii* IgG antibodies with the commercially available enzyme immunoassay kit "*Toxoplasma* IgG" (Diagnostic Automation Inc., Woodland Hills, CA, USA). Levels of anti-*T. gondii* IgG antibody were expressed as International Units (IU)/mL, and a result ≥ 8 IU/mL was considered positive. Serum samples with anti-*T. gondii* IgG antibodies were further tested for anti-*T. gondii* IgM antibodies by the commercially available enzyme immunoassay "*Toxoplasma* IgM" kit (Diagnostic Automation Inc.). All assays were performed according to the manufacturer's instructions. Positive and negative controls included in the kits were included in each run.

Extraction of DNA and detection of *T. gondii* DNA

Miners with *Toxoplasma*-specific IgG antibodies by EIA were further examined for *T. gondii* DNA by nested-polymerase chain reaction (PCR). Extraction of DNA from whole blood was performed following a protocol described by Iranpour and Esmailizadeh (http://www.protocol-online.org/prot/Protocols/Rapid-Extraction-of-High-Quality-DNA-from-Whole-Blood-Stored-at-4-C-for-Long-Period-4175.html). PCR amplification was performed with primers directed against the B1 gene of *T. gondii* and following the protocol described by Roth et al [11]. Amplification products were analyzed with 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized by ultraviolet transillumination.

Statistical analysis

Results were analyzed with the aid of the software Microsoft Excel 2010, Epi Info version 7 (Centers for Disease Control and Prevention: http://wwwn.cdc.gov/epiinfo/) and SPSS version 15.0 (SPSS Inc. Chicago, IL). For calculation of the sample size, we used a 95% confidence level, a power of 80%, a 1:2 proportion of cases and controls, a reference seroprevalence of 23.8% [10] as the expected frequency of exposure in controls, and an odds ratio (OR) of 2. The result of the sample size calculation was 115 cases and 229 controls. We compared the age of cases and controls using the Student's *t*-test. The Pearson's Chi-square test and the Fisher exact test (when values were small) were used to determine the association of *T. gondii* seropositivity with the characteristics of miners. As a strategy for multivariate analysis, only characteristics of miners with a P value ≤ 0.10 obtained in the bivariate analysis were included in the analysis. OR and 95% confidence interval (CI) were calculated using logistic regression analysis with the Enter method. A P value < 0.05 was considered as statistically

Table 1. Socio-Demographic and Housing Characteristics of Miners and Prevalence of *T. gondii* Infection

Characteristics	Total subjects	Prevalence of *T. gondii* infection		P value
		No.	%	
Age groups (years)				
30 or less	26	16	61.5	0.73
31 - 50	59	37	62.7	
> 50	40	22	55.0	
Birth place				
Durango State	98	64	65.3	0.02
Other Mexican State	27	11	40.7	
Educational level				
No education	16	9	56.3	0.72
1 - 6 years	68	42	61.8	
7 - 12 years	36	20	55.6	
> 12 years	5	4	80.0	
Socio-economic level				
Low	61	42	68.9	0.04
Medium	64	33	51.6	
Flooring at home				
Ceramic or wood	5	3	60.0	0.87
Concrete	92	54	58.7	
Soil	28	18	64.3	
Availability of potable water				
In home	2	1	50.0	0.34
In land	3	3	100.0	
In street	120	71	59.2	
Toilet facilities				
Sewage pipes	5	3	60.0	1.00
Latrine or another	120	72	60.0	
Crowding at home				
No	41	26	63.4	0.05
Semi-crowded	55	27	49.1	
Overcrowded	29	22	75.9	
Education of the head of family				
7 years or more	17	10	58.8	0.52
4 - 6 years	53	29	54.7	
Up to 3 years	55	36	65.5	

significant.

Ethics statement

The Ethical Committee of the General Hospital of the Secretary of Health in Durango City approved this project. The purpose and procedures of this study were explained to all miners examined. A written informed consent was obtained from each participant.

Results

Anti-*T. gondii* IgG antibodies were detected in 75 (60.0%) of 125 miners and in 55 (22.0%) of 250 controls. Seroprevalence of anti-*T. gondii* IgG antibodies was significantly higher in miners than in controls (OR = 5.31; 95% CI: 3.33 - 8.47;

Table 2. Bivariate Analysis of Selected Behavioral Characteristics and Infection With *T. gondii* in Miners

Characteristics	No. of subjects tested	Prevalence of *T. gondii* infection		P value
		No.	%	
Cleaning cat excrement				
Yes	43	21	48.8	0.06
No	82	54	65.9	
Raising farm animals				
Yes	99	61	61.6	0.47
No	26	14	53.8	
National trips				
Yes	77	43	55.8	0.23
No	48	32	66.7	
Goat meat consumption				
Yes	101	56	55.4	0.03
No	24	19	79.2	
Sheep meat consumption				
Yes	59	30	50.8	0.04
No	66	45	68.2	
Boar meat consumption				
Yes	94	61	64.9	0.05
No	31	14	45.2	
Chicken meat consumption				
Yes	124	75	60.5	0.40
No	1	0	0.0	
Turkey meat consumption				
Yes	65	32	49.2	0.01
No	60	43	71.7	
Pigeon meat consumption				
Yes	72	48	66.7	0.07
No	53	27	50.9	
Quail meat consumption				
Yes	39	26	66.7	0.30
No	86	49	57.0	
Rabbit meat consumption				
Yes	51	27	52.9	0.18
No	74	48	64.9	
Armadillo meat consumption				
Yes	60	38	63.3	0.46
No	65	37	56.9	
Iguana meat consumption				
Yes	24	16	66.7	0.45
No	101	59	58.4	
Badger meat consumption				
Yes	24	17	70.8	0.22
No	101	58	57.4	

Table 2. Bivariate Analysis of Selected Behavioral Characteristics and Infection With *T. gondii* in Miners - *(continued)*

Characteristics	No. of subjects tested	Prevalence of *T. gondii* infection		P value
		No.	%	
Degree of meat cooking				
Undercooked	6	5	83.3	0.39
Well done	117	68	58.1	
Raw dried meat				
Yes	85	46	54.1	0.06
No	39	28	71.8	
Chorizo consumption				
Yes	124	75	60.5	0.40
No	1	0	0.0	
Brain of cow consumption				
Yes	48	24	50.0	0.07
No	77	51	66.2	
Unwashed raw vegetables				
Yes	85	54	63.5	0.24
No	40	21	52.5	
Untreated water				
Yes	107	66	61.7	0.34
No	18	9	50.0	
Frequency of eating out of home				
Never	1	1	100.0	0.19
1 - 10 times a year	84	46	54.8	
> 10 times a year	40	28	70.0	
Alcohol consumption				
Yes	67	45	67.2	0.07
No	58	30	51.7	
Tobacco smoking				
Yes	57	39	68.4	0.07
No	68	36	52.9	

P < 0.001). Of the 75 anti-*T. gondii* IgG positive miners, 30 (40.0%) had anti-*T. gondii* IgG antibody levels higher than 150 IU/mL, 10 (13.3%) between 100 and 150 IU/mL, and 35 (46.7%) between 8 and 99 IU/mL. All seropositive controls had ≥ 8 IU/mL of anti-*T. gondii* IgG antibodies as determined by the qualitative test. However, we were unable to quantitate further for the specific IgG level in all 55 seropositive controls. IgG levels could be determined in only 36 of 55 seropositive controls; of these 27 (75.0%) had anti-*T. gondii* IgG antibody levels higher than 150 IU/mL, two (5.6%) between 100 and 150 IU/mL, and seven (19.4%) between 8 and 99 IU/mL. The frequency of individuals with high IgG levels was significantly higher in the controls compared to the cases group (P = 0.003).

Of the 75 miners seropositive for anti-*T. gondii* IgG antibodies, 39 (52.0%) were also positive for anti-*T. gondii* IgM antibodies compared to only eight (14.5%) of the 55 controls seropositive to anti-*T. gondii* IgG antibodies (P < 0.001). DNA of *T. gondii* was detected in 13 miners, and eight (61.5%) of them were positive for anti-*T. gondii* IgM antibodies.

The frequency of IgG was similar (P = 0.47) in participants working less than 1 year as a miner (62.5%) and those with 1 - 5 years (68.8%) or > 5 years (56.5%); the frequency of IgM was comparable (P = 0.92) in participants with less than 1 year of working as a miner (25.0%), those with 1 - 5 years (31.3%) or those with > 5 years (31.8%).

Concerning socio-demographic and housing characteristics (Table 1), bivariate analysis showed three characteristics potentially (P values ≤ 0.10) associated with *T. gondii* infection: birth place (P = 0.02), socioeconomic status (P = 0.04), and crowing at home (P = 0.05). Other socio-demographic and housing characteristics of miners including age, educational level, flooring at home, availability of potable water, form of elimination of excretes, and years of education of the head of

Table 3. Multivariate Analysis of Selected Characteristics of Miners and Their Association With *T. gondii* Infection

Characteristics	Odds ratio	95% confidence interval	P value
Birth place (Durango State)	3.44	1.09 - 10.7	0.03
Socioeconomic level (low)	2.05	0.73 - 5.71	0.17
Cleaning cat excrement (yes)	0.33	0.11 - 0.90	0.03
Goat meat consumption (yes)	0.16	0.03 - 0.76	0.02
Sheep meat consumption (yes)	1.10	0.34 - 3.48	0.88
Boar meat consumption (yes)	5.53	1.49 - 20.3	0.01
Turkey meat consumption (yes)	0.35	0.12 - 1.00	0.05
Pigeon meat consumption (yes)	2.20	0.80 - 5.98	0.12
Raw dried meat (yes)	0.45	0.15 - 1.32	0.15
Brain of cow consumption (yes)	0.50	0.18 - 1.32	0.16
Alcohol consumption (yes)	0.83	0.27 - 2.51	0.75
Tobacco use (yes)	2.40	0.85 - 6.73	0.10
Crowding			
Semi-crowded	2.78	0.86 - 8.93	0.09
Overcrowded	5.83	1.49 - 22.8	0.01

the family had P values > 0.10.

With respect to the clinical characteristics, all seropositive miners referred themselves as healthy. The frequency of *T. gondii* seropositivity was higher in miners without memory impairment (56/85, 65.9%) than in miners with memory impairment (18/39, 46.2%) (P = 0.03). Other clinical characteristics including history of lymphadenopathy, surgery, blood transfusion, impairments in reflexes, hearing and vision, frequent abdominal pain or headache, and dizziness showed no association with seropositivity. None of the miners had received an organ transplant.

Of the behavioral characteristics of the miners examined (Table 2), 10 variables had P values ≤ 0.10 in the bivariate analysis: cleaning cat excrement (P = 0.06), consumption of meat from goat (P = 0.03), sheep (P = 0.04), boar (P = 0.05), turkey (P = 0.01), and pigeon (P = 0.07), consumption of raw dried meat (P = 0.06), cow's brains (P = 0.07), alcohol consumption (P = 0.07), and tobacco smoking (P = 0.07).

Multivariate analysis of socio-demographic, housing, and behavioral characteristics of miners with P values ≤ 0.10 in the bivariate analysis (Table 3) showed that *T. gondii* seropositivity was positively associated with being born in Durango State (OR = 3.44; 95% CI: 1.09 - 10.7; P = 0.03), consumption of boar meat (OR = 5.53; 95% CI: 1.49 - 20.3; P = 0.01), and overcrowded homes (OR = 5.83; 95% CI: 1.49 - 22.8; P = 0.01), and seropositivity was negatively associated with cleaning cat excrement (OR = 0.33; 95% CI: 0.11 - 0.90; P = 0.03), and consumption of goat meat (OR = 0.16; 95% CI: 0.03 - 0.76; P = 0.02).

Discussion

Very little is known about the epidemiology of *T. gondii* infection in miners. To the best of our knowledge, the correlation of *T. gondii* infection with the occupation of miner has not been assessed by an age- and gender-matched case-control study design. Therefore, we performed an age- and gender-matched case-control study to investigate the association of *T. gondii* infection with the occupation of miner in the northern Mexican State of Durango.

Remarkably, we found that the prevalence of *T. gondii* exposure was significantly higher in miners than in controls. The seroprevalence found in miners in Durango, Mexico is higher than those reported in miners in other countries. A 7.7% prevalence of infection was found in coal miners in China using the indirect hemagglutination test [12]. In Ukraine, 37.7% of miners were seropositive for *T. gondii* using complement-fixation, passive hemagglutination, and intradermal toxoplasmin tests [13]. Furthermore, the high seroprevalence of *T. gondii* exposure found in miners (60.0%) is the highest seroprevalence reported in population groups in Durango State so far. Thus, seroprevalence found in miners is higher than the seroprevalences of *T. gondii* infection reported in adults in rural communities in Durango State (23.8%) [10], in schizophrenic patients (20%) [14], in waste pickers (21.1%) [15], inmates (21.1%) [16], and ethnic groups living in rural communities including Tepehuanos (22.4%) [17] and Huicholes (33.2%) [18]. In addition, the seroprevalence found in miners is higher than the weighted mean (19.27%) national seroprevalence of *T. gondii* infection found in Mexico [19]. It is not clear why miners had a higher seroprevalence of *T. gondii* exposure than age- and gender-matched controls which were also obtained from rural settings. Seroprevalence of infection with *T. gondii* increases with age as reported in general populations in rural [10] and urban [20] Durango. However, in the present study, no such increase in *T. gondii* exposure with age was observed, and a surprisingly high (61.5%) seroprevalence of *T. gondii* infec-

tion was already observed in the youngest miners aged 18 - 30 years old.

We searched for socio-demographic, work, housing and behavioral characteristics to investigate the high seroprevalence of *T. gondii* in miners. Duration in the activity did not correlate with *T. gondii* exposure. Even miners with less than 1 year as a miners had a high seroprevalence of infection with *T. gondii*. Multivariate analysis showed that *T. gondii* exposure was positively associated with being born in Durango State. This result was unexpected since *T. gondii* exposure has been repeatedly associated with the characteristic of being born out of Durango State in diverse cohorts including the general population in Durango City [20], inmates [16], patients with vision and hearing impairments, cancer, HIV, or undergoing hemodialysis [21], female sex workers [22], elderly people [23], patients with heart diseases [24], and people applying for medical certificates [25]. The association of *T. gondii* infection with being born in Durango State found in this study likely indicates that infection was acquired in Durango State. In fact, traveling did not increase the prevalence of infection with *T. gondii* in miners. Furthermore, multivariate analysis showed that infection with *T. gondii* was associated with consumption of boar meat, and living in an overcrowded home. These characteristics may have contributed to the high seroprevalence of *T. gondii* infection in miners. Consumption of boar meat was associated with *T. gondii* seropositivity in several populations in the region with lower seroprevalence than miners including patients with work accidents [26], elderly people [23], and the general population in Durango City [20]. Concerning the association of infection with *T. gondii* and living in an overcrowded home, this is the first time we found such association in our studies of infection with *T. gondii* in the region. Living in an overcrowded area has been considered as a contributing factor for infection with *T. gondii* in pregnant women in Nigeria [27]. It is not clear why overcrowding influenced the seroprevalence of *T. gondii* infection in that study, but other factors for infection including poor sanitation and contamination of environment with cat excrement were also present [27]. In addition, in the Third National Health and Nutrition Examination Survey in the USA (1988 - 1994), researchers found that risk for *T. gondii* infection increased in those who lived in crowded conditions [28]. On the other hand, we found that infection with *T. gondii* was negatively associated with cleaning cat excrement, and consumption of goat meat. These factors have been suggested as risks for infection with *T. gondii* exposure by others [29, 30].

The present study has some limitations. The sample size is small, and we studied miners working in only one mine. Further studies should include a larger sample size and sample miners of more than one mine. A high frequency (61.5%) of positive *T. gondii* PCR assays among miners seropositive for anti-*T. gondii* IgM antibodies was found. Therefore, further research on the epidemiology of acute cases of *T. gondii* infection in miners should be conducted.

We conclude that miners represent a risk group for infection with *T. gondii*. This is the first age- and gender-matched study on the association of *T. gondii* infection and the occupation of miner. Further studies to confirm our results are needed.

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INFLUENCE OF *TOXOPLASMA GONDII* INFECTION ON SYMPTOMS AND SIGNS OF PREMENSTRUAL SYNDROME: A CROSS-SECTIONAL STUDY

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Infection with *Toxoplasma gondii* in brain may cause some symptoms that resemble those in women with premenstrual syndrome. To determine the association of *T. gondii* infection with symptoms and signs of premenstrual syndrome, we examined 489 women aged 30–40 years old. Sera of participants were analyzed for the presence of anti-*Toxoplasma* IgG and IgM antibodies using enzyme-linked immunoassays (EIA) and *T. gondii* DNA by polymerase chain reaction (PCR).

Anti-*T. gondii* IgG antibodies were found in 38 (7.8%) of the women studied. Anti-*T. gondii* IgM antibodies were found in 13 (34.2%) of the 38 IgG seropositive women. Logistic regression showed two variables associated with seropositivity to *T. gondii:* presence of diarrhea (odds ratio [OR] = 6.10; 95% confidence interval [CI]: 1.37–27.85; $P = 0.01$) and weight gain (OR = 2.89; 95% CI: 1.37–6.07; $P = 0.005$), and two variables associated with high (>150 IU/ml) levels of IgG against *T. gondii:* presence of diarrhea (OR = 7.40; 95% CI: 1.79–30.46; $P = 0.006$) and abdominal inflammation (OR = 3.38; 95% CI: 1.13–10.10; $P = 0.02$). Positivity to EIA IgG and PCR was positively associated with obesity and negatively associated with joint pain by bivariate analysis.

Our study for the first time reveals a potential association of *T. gondii* infection with clinical manifestations of premenstrual syndrome.

Keywords: *Toxoplasma gondii,* seroprevalence, premenstrual syndrome, cross-sectional study

Introduction

It is estimated that about one third of humanity is infected with the protozoan parasite *Toxoplasma gondii* [1, 2]. Infection with *T. gondii* is zoonotic, and it is most frequently acquired by the ingestion of raw or undercooked meat of *T. gondii*-infected animals containing tissue cysts, or ingestion of food or water contaminated with *T. gondii* oocysts shed by cats [3, 4]. Other routes of *T. gondii* infection are vertical [5], organ transplantation [6], and blood transfusion [7]. Most infections with *T. gondii* are asymptomatic [3]. However, some infected individuals develop clinical manifestations of the disease called toxoplasmosis [5]. Individuals suffering from toxoplasmosis may have involvement of lymph nodes, eyes, or central nervous system [3, 8]. A life-threatening toxoplasmosis

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may occur in immunocompromised patients [9]. Infection with *T. gondii* has been linked to psychiatric illnesses, i.e., schizophrenia [10, 11], obsessive–compulsive disorder [10], intermittent explosive disorder [12], depression [13], and generalized anxiety disorder [14]. A number of general symptoms of toxoplasmosis have been described including headache [15, 16], pain and weakness of muscles [16–18], fatigue [16, 19, 20], difficulty concentrating [19], and confusion [13].

Premenstrual syndrome is characterized by recurrent affective, physical, and behavioral symptoms that develop during the luteal menstrual cycle and disappear within a few days of menstruation [21, 22]. A severe form of this syndrome is called premenstrual dysphoric disorder [22, 23]. Clinical manifestations of premenstrual syndrome include fatigue, impaired concentration [24, 25], confusion [24], headache [26], and depression [27]. These clinical features are also observed in toxoplasmosis. It is possible that infection with *T. gondii* in brain might cause or influence some symptoms in women during the premenstrual period. In a recent study, infection with *T. gondii* was associated with out of control feeling or overwhelmed in women suffering from premenstrual dysphoric disorder [28]. However, it is unknown whether *T. gondii* infection might influence symptoms in premenstrual syndrome. Symptoms of premenstrual syndrome might be not only hormonal but also nervous in nature. Infection with *T. gondii* in brain might be linked to clinical manifestations in premenstrual syndrome as occurred in menopause [29]. Since the link of *T. gondii* infection and clinical manifestations of premenstrual syndrome has not been investigated in the past, we investigated the association of *T. gondii* infection with clinical characteristics of premenstrual syndrome in women in the northern Mexican city of Durango.

Materials and methods

Study design and study population

We performed a cross-sectional study of 489 women who attended general consultations in two public primary healthcare centers: Centro de Salud #2 of the Secretary of Health (n = 327) and Clinic of Family Medicine of the Institute of Security and Social Services of State Workers (n = 162) in Durango City, Mexico. All women were examined from February to April 2016. Inclusion criteria for enrollment were women aged 30–40 years old who accepted to participate in the study. Socioeconomic status and occupation of the women were not restrictive criteria for enrollment. Pregnancy was an exclusion criterion. Mean age in women studied was 35.27 ± 3.47.

Clinical characteristics of women

We used a face-to-face questionnaire to record the symptoms and signs of premenstrual syndrome in the women studied. Clinical data studied were presence of irregular periods, severity of menstruation, suffering from mental illness, vaginal infections, thyroid disease, obesity, arterial hypertension, sleep problems, fatigue, memory lapses, difficulty concentrating, confusion, judgment problems, mood changes, low self-esteem, depression, guilty feeling, increase of fears, panic attacks, anxiety, tension, nervousness, irritability, aggressiveness, lack of interest in daily activities, lack of interest in social relations, out of control feeling or overwhelmed, reduced tolerance to noises and lights, dizziness, headache, migraine, allergy, breast pain, bouts of rapid heartbeat, decrease in muscle power, joint pain, low back pain, muscle tension, clumsiness, tingling extremities, electric shock sensation, bruises, painful periods, edema in ankles, hands or feet, decreased libido, increased libido, dyspareunia, abdominal bloating, gas, abdominal pain, constipation, diarrhea, nauseas, abdominal inflammation, appetite disturbance, desire to eat certain food or eat a lot, weight gain, presence of acne, presence of herpes labialis, and respiratory problems.

*Detection of anti-*T. gondii *antibodies*

We obtained a serum sample from each woman. Sera were frozen at −20 °C until analyzed. Anti-*T. gondii* IgG antibodies were detected in sera with the commercially available enzyme immunoassay (EIA) kit "*Toxoplasma* IgG" (International Immuno-Diagnostics, Foster City, CA, USA). Anti-*T. gondii* IgG antibody levels were expressed as International Units (IU)/ml, and a cut off of ≥ 8 IU/ml was used for seropositivity. Sera with anti-*T. gondii* IgG antibodies were further analyzed for anti-*T. gondii* IgM antibodies by the commercially available EIA "*Toxoplasma* IgM" kit (Diagnostic Automation Inc., Calabasas, CA, USA). All assays were performed according to the manufacturer's instructions.

DNA extraction and T. gondii *polymerase chain reaction*

Women with positive EIA for *T. gondii* IgG antibodies were further examined to detect *T. gondii* DNA by nested polymerase chain reaction (PCR). Extraction of DNA was performed from whole blood according to a protocol described by Iranpour and Esmailizadeh (http://www.protocol-online.org/prot/Protocols/Rapid-Extraction-of-High-Quality-DNA-from-Whole-Blood-Stored-at-4-C-for-Long-Period-4175.html). We used a PCR protocol and two pairs of primer directed against the B1 gene of *T. gondii* as described elsewhere [30]. This protocol was previously tested and showed high specificity and sensitivity: 0.01 to 0.02 fg of the target DNA in the presence of 1 µg of contaminating negative human DNA was detected by PCR [31]. PCR products were electrophoresed with agarose gels, stained with ethidium bromide, and visualized by ultraviolet transillumination.

Statistical analysis

Analysis of results was performed by using the following software: SPSS 15.0 (SPSS Inc. Chicago, Illinois), Microsoft Excel, and Epi Info 7. For calculation of the sample size, we used: a) a reference seroprevalence of 6.1% [32] as the expected frequency for the factor under study, b) 200,000 as the population size from which the sample was selected, c) a 2.2% of confidence limits, and d) a 95% confidence level. The result of the sample size calculation was 454 subjects. We assessed the association of *T. gondii* seropositivity and the clinical characteristics of women with the Pearson's chi-squared test or the two-tailed Fisher's exact test (when values were small). We included in the multivariate analysis only variables with a *P* value ≤0.10 obtained in the bivariate analysis. Odds ratio (OR) and 95% confidence interval (CI) were calculated by multivariate analysis using logistic regression with the Enter method. Statistical significance was set at a *P* value <0.05.

Ethical aspects

This study was approved by the Ethics Committee of the General Hospital of the Secretary of Health in Durango City, Mexico. Participation was voluntary, and the purpose and procedures of this study were explained to all participants. Furthermore, a written informed consent was obtained from each participant.

Results

Anti-*T. gondii* IgG antibodies were found in 38 (7.8%) of the 489 women studied. Of the 38 anti-*T. gondii* IgG positive women, 22 (57.9%) had IgG levels >150 IU/ml, 1 (2.6%) between 100 and 150 IU/ml, and 15 (39.5%) between 8 and 99 IU/ml. Anti-*T. gondii* IgM antibodies were found in 13 (34.2%) of the 38 IgG seropositive women. DNA of *T. gondii* was detected in six (15.8%) of the 38 women with IgG antibodies against *T. gondii*.

Seropositive women showed from four to 41 (mean: 15.1 ± 8.5) signs or symptoms of premenstrual syndrome. Seronegative women (n = 451) had from one to 45 (mean: 16.7 ± 9.1) signs or symptoms of premenstrual syndrome. Mean number of signs or symptoms in seropositive women was similar to that found in seronegative women (P = 0.28).

Bivariate analysis of clinical characteristics of premenstrual syndrome and IgG seropositivity to *T. gondii* showed ten variables with a *P* value less than 0.10: confusion, allergy, low back pain, tingling extremities, electric shock sensation, increased libido, abdominal bloating, gas, diarrhea, and weight gain. Other clinical characteristics of premenstrual syndrome showed *P* values equal to or higher than 0.10 by bivariate analysis. *Table 1* shows results of bivariate analysis of a selection of clinical data of premenstrual syndrome and IgG seropositivity to *T. gondii*. Further analysis by logistic regression of variables with *P* value less than 0.10 obtained by bivariate analysis

Table 1. Results of bivariate analysis of a selection of premenstrual clinical characteristics of women and IgG seropositivity to *T. gondii*

	Women tested	Prevalence of *T. gondii* infection		*P* value
Characteristic	No.	No.	%	
Obesity				
Yes	192	18	9.4	0.28
No	297	20	6.7	
Arterial hypertension				
Yes	46	6	13.0	0.14
No	441	31	7.0	
Confusion				
Yes	99	3	3	0.04
No	389	35	9	
Irritability				
Yes	240	15	6.3	0.21
No	249	23	9.2	
Reduced tolerance to noises and lights				
Yes	131	6	4.6	0.11
No	358	32	8.9	
Dizziness				
Yes	172	9	5.2	0.12
No	317	29	9.1	

Table 1. *(cont'd)*

	Women tested	Prevalence of *T. gondii* infection		*P* value
Characteristic	No.	No.	%	
Headache				
Yes	278	23	8.3	0.63
No	211	15	7.1	
Migraine				
Yes	124	7	5.6	0.30
No	365	31	8.5	
Allergy				
Yes	118	5	4.2	0.10
No	371	33	8.9	
Breast pain				
Yes	234	15	6.4	0.28
No	255	23	9	
Bouts of rapid heart beat				
Yes	115	5	4.3	0.11
No	373	33	8.8	
Decrease in muscle power				
Yes	176	10	5.7	0.19
No	313	28	8.9	
Joint pain				
Yes	217	13	6	0.23
No	270	24	8.9	
Low back pain				
Yes	308	18	5.8	0.03
No	181	20	11	
Tingling extremities				
Yes	200	10	5	0.05
No	289	28	9.7	
Electric shock sensation				
Yes	131	5	3.8	0.04
No	358	33	9.2	
Edema in ankles, hands, or feet				
Yes	111	7	6.3	0.49
No	375	31	8.3	
Decreased libido				
Yes	151	9	6	0.30
No	334	29	8.7	
Increased libido				
Yes	34	0	0	0.09
No	449	38	8.5	
Abdominal bloating				
Yes	108	6	5.6	0.00
No	381	32	8.4	
Gas				
Yes	30	5	16.7	0.07
No	459	33	7.2	

Table 1. *(cont'd)*

	Women tested	Prevalence of *T. gondii* infection		*P* value
Characteristic	No.	No.	%	
Constipation				
Yes	123	6	4.9	0.16
No	366	32	8.7	
Diarrhea				
Yes	14	3	21.4	0.08
No	475	35	7.4	
Abdominal inflammation				
Yes	48	6	12.5	0.24
No	441	32	7.3	
Weight gain				
Yes	219	23	10.5	0.03
No	267	14	5.2	

showed that only two variables were associated with seropositivity to *T. gondii*: presence of diarrhea (OR = 6.10; 95% CI: 1.37–27.85; $P = 0.01$) and weight gain (OR = 2.89; 95% CI: 1.37–6.07; $P = 0.005$) *(Table 2)*.

Bivariate analysis of clinical characteristics of premenstrual syndrome and high (>150 IU/ml) IgG levels to *T. gondii* showed only six variables with a *P* value less than 0.10: low self-esteem, irritability, low back pain, tingling extremities, diarrhea, and abdominal inflammation. Further analysis by logistic regression of these variables with *P* values less than 0.10 obtained by bivariate analysis showed that two variables were associated with high levels of IgG against *T. gondii*: presence of diarrhea (OR = 7.40; 95% CI: 1.79–30.46; $P = 0.006$) and abdominal inflammation (OR = 3.38; 95% CI: 1.13–10.10; $P = 0.02$) *(Table 3)*.

With respect to the association of premenstrual clinical manifestations and seropositivity of both IgG and IgM anti-*T. gondii,* bivariate analysis showed no significant associations, and only the variables tingling extremities and diarrhea showed borderline ($P = 0.05$) associations.

Table 2. Multivariate analysis of selected premenstrual clinical characteristics of women and their association with *T. gondii* infection

Characteristic	Odds ratio	95% confidence interval	*P* value
Confusion	0.38	0.11–1.34	0.13
Allergy	0.50	0.18–1.38	0.18
Low back pain	0.48	0.23–1.02	0.05
Tingling extremities	0.78	0.33–1.86	0.58
Electric shock sensation	0.52	0.17–1.58	0.25
Abdominal bloating	0.89	0.33–2.34	0.81
Gas	2.60	0.79–8.57	0.11
Diarrhea	6.10	1.37–27.85	0.01
Weight gain	2.89	1.37–6.07	0.005

Table 3. Multivariate analysis of selected premenstrual characteristics and their association with high (>150 IU/ml) levels of IgG to *T. gondii*

Characteristic	Odds ratio	95% confidence interval	*P* value
Low self-esteem	0.58	0.19–1.74	0.33
Irritability	0.67	0.24–1.86	0.44
Low back pain	0.53	0.20–1.36	0.18
Tingling extremities	0.62	0.21–1.81	0.38
Diarrhea	7.40	1.79–30.46	0.006
Abdominal inflammation	3.38	1.13–10.10	0.02

To avoid bias and due to a small number of cases with IgM seropositivity, no further regression analysis with these variables was performed.

Concerning the results of the positivity to both IgG antibodies against *T. gondii* and DNA of *T. gondii* by PCR, women with obesity showed a significantly ($P = 0.03$) higher prevalence of *T. gondii* (5/192: 2.6%) than women without obesity (1/297: 0.3%) whereas women with joint pain showed a significantly ($P = 0.03$) lower prevalence of *T. gondii* (0/217) than women without joint pain (6/270: 2.2%).

Discussion

Premenstrual syndrome has a number of signs and symptoms also observed in toxoplasmosis. Therefore, we hypothesized that *T. gondii* infection may have an influence on clinical manifestations of premenstrual syndrome. As far as we know, the association between *T. gondii* infection and signs and symptoms of premenstrual syndrome has not been assessed yet. Therefore, this study aimed to determine whether infection with *T. gondii* was associated with clinical characteristics of premenstrual syndrome in women in Durango City, Mexico. We found that women seropositive for *T. gondii* had a similar mean number of signs or symptoms of premenstrual syndrome than seronegative women. Results suggest that infection with *T. gondii* does not influence on the number of clinical manifestations of premenstrual syndrome in general. However, logistic regression showed in particular that infection with *T. gondii* is associated with specific clinical characteristics of premenstrual syndrome. Thus, results suggest that *T. gondii* infection may influence qualitatively on clinical manifestations of premenstrual syndrome. Remarkably, both IgG seropositivity to *T. gondii* and high levels of IgG against *T. gondii* were associated with the presence of diarrhea. It is not clear why infection with *T. gondii* was associated with diarrhea during the premenstrual period. Diarrhea is a well-known clinical sign included within the physical features of premenstrual syndrome [33]. In a Chinese study about prevalence of premenstrual syndrome in women at reproductive age, researchers found diarrhea as the fourth most frequent clinical characteristic in premenstrual syndrome just after irritation, depression, and anxiety [33]. In addition, in a prevalence study about the menstrual cycle and its effect on inflammatory bowel disease and irritable bowel syndrome, Kane et al. found that women with Crohn's disease were more likely to report increased gastrointestinal symptoms during menstruation, being diarrhea the clinical feature reported most often [34]. In a recent study, Zhang et al. reported that Chinese women suffering from both diarrhea-predominant irritable bowel syndrome and premenstrual syndrome had more severe bowel symptoms [35]. On the other hand, infection with *T. gondii* may lead to diarrhea in humans and animals [36]. Presence of diarrhea in *T. gondii* infected individuals has been unusually reported. However, the link between infection with *T. gondii* and diarrhea in humans has been scantly studied. A case of gastric toxoplasmosis with diarrhea in a man with acquired immunodeficiency syndrome was reported [37]. Similarly, a case of toxoplasmic colitis with diarrhea where microorganisms were identified in the colonic mucosa and confirmed by immunohistochemistry was reported [38]. In animals, severe or fatal toxoplasmosis cases with diarrhea have been reported in cats [39, 40] and a valley quail [41]. It is unclear how frequent diarrhea occurs in immunocompromised and immunocompetent subjects. We may hypothesize that *T. gondii* may affect intestines of women during the premenstrual period perhaps under a hormonal influence leading to diarrhea. It is also possible that *T. gondii* causes diarrhea by affecting enteric neurons. Experiments in rats have shown that infection with *T. gondii* causes changes in myenteric neurons of the jejunum, i.e., atrophy of myenteric neurons along with increased weight gain in rats at 30 days of infection, or hypertrophy of myenteric neurons along with normal weight gain in rats at 90 days after infection [36]. Interestingly, IgG seropositivity to *T. gondii* was associated with weight gain. It is not clear why women who have gained weight had a higher seroprevalence of *T. gondii* infection that those without weight gain. Experimental infections of *T. gondii* have showed weight gain in rats after 30 days of infection [36]. In humans, *T. gondii* infection has been associated with weight gain in pregnant women [42]. In addition, both *T. gondii* seroprevalence and high IgG anti-*T. gondii* antibody levels have been associated with obesity [43]. In fact, results of the positivity to both IgG antibodies against *T. gondii* and DNA of *T. gondii* in this study showed that women with obesity had a significantly higher prevalence of *T. gondii* than women without obesity. On the other hand, women with joint pain showed a significantly lower prevalence of *T. gondii* than women without joint pain. This finding suggests that *T. gondii* was not an important factor for joint pain in the women studied.

Logistic regression analysis also showed that high levels of IgG against *T. gondii* were associated with abdominal inflammation. *T. gondii* may cause inflammation in organs and tissues in the abdomen, as observed in experimental infections in mice [44]. Therefore, the presence of this clinical feature may suggest an active immune reaction against *T. gondii* in abdomen.

Limitations of our study included small sample size of the women studied and a low prevalence of IgG, IgM, and PCR positivity. However, strengths of our study include that women were studied from two health centers of Durango City and that we used detection of DNA of *T. gondii* to increase the evidence of *T. gondii* exposure.

Conclusions

The present study for the first time points towards an association of *T. gondii* infection with clinical manifestations of premenstrual syndrome, i.e., physical symptoms. Re-

sults warrant further research of the role of *T. gondii* on clinical manifestations of premenstrual syndrome.

Competing interests

The authors declare that no competing interests exist.

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Original Article

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Toxoplasma gondii Infection and Premenstrual Dysphoric Disorder: A Cross-Sectional Study

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Abstract

Background: Premenstrual dysphoric disorder is a severe form of premenstrual syndrome. The influence of *Toxoplasma gondii (T. gondii)* infection on clinical features in women with this disorder has not been studied. Therefore, we determined the association of *T. gondii* infection with symptoms and signs in women suffering from premenstrual dysphoric disorder.

Methods: We performed a cross-sectional study of 151 women suffering from premenstrual dysphoric disorder. Anti-*Toxoplasma* IgG and IgM antibodies were detected in sera of the participants using enzyme-linked immunoassays (EIAs). In addition, *T. gondii* DNA was detected in whole blood of IgG seropositive participants using polymerase chain reaction. We obtained the clinical data of women with the aid of a questionnaire. The association of *T. gondii* infection with clinical characteristics of women was assessed by bivariate and multivariate analyses.

Results: Anti-*T. gondii* IgG antibodies were found in 10 (6.6%) of the 151 women studied. Of the 10 IgG seropositive women, four (40.0%) were positive for anti-*T. gondii* IgM antibodies, and one (10.0%) for *T. gondii* DNA. Mean number (25.8 ± 7.58) of premenstrual clinical characteristics in seropositive women was similar to that (29.22 ± 9.13) found in seronegative women (P = 0.25). Logistic regression showed that seropositivity to *T. gondii* was negatively associated with difficulty concentrating (OR: 0.18; 95% CI: 0.03 - 0.91; P = 0.03), and positively associated with out of control feeling or overwhelmed (OR: 9.00; 95% CI: 1.32 - 62.00; P = 0.02).

Conclusions: Results of this first study on the association of *T. gondii* infection and clinical characteristics of premenstrual dysphoric disorder suggest that this infection might be linked to some symptoms of this disorder. We report for the first time the association of *T. gondii* infection and out of control feeling or overwhelmed. Results warrant for further research on the role of *T. gondii* in premenstrual dysphoric disorder.

Keywords: *Toxoplasma gondii*; Seroprevalence; Premenstrual dysphoric disorder; Cross-sectional study

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Introduction

The coccidian parasite *Toxoplasma gondii (T. gondii)* causes infections in humans worldwide [1]. Cats are the definitive host of *T. gondii*, whereas humans and other warm-blooded animals are intermediate hosts [2]. Most infections with *T. gondii* in humans are acquired by ingestion of food or water contaminated with *T. gondii* oocysts shed by cats, or by the ingestion of raw or undercooked meat containing tissue cysts [3]. Less frequently, infection with *T. gondii* may occur by organ transplantation [4], and blood transfusion [5]. In addition, primary infection with *T. gondii* during pregnancy may lead to vertical transmission and congenital disease [3, 6]. Infections with *T. gondii* are usually asymptomatic [3]. Subjects with clinical manifestations of infection (toxoplasmosis) may present with disease in eyes, lymph nodes and central nervous system [3, 7, 8]. Toxoplasmosis is particularly severe in immunocompromised individuals [9]. Common symptoms of toxoplasmosis include fatigue, headache, muscle aches, and difficulty concentrating [10]. Furthermore, infection with *T. gondii* has been associated with a number of psychiatric disorders including depression [11], schizophrenia [11, 12], im-

pulsive aggression [13], generalized anxiety disorder [14], and suicide attempts [15].

Premenstrual dysphoric disorder is a severe form of premenstrual syndrome with serious psychological symptoms [16]. This disorder is characterized by cognitive-affective symptoms that appear in a cyclic manner during the premenstrual period [17]. This illness has been recently designated as a disorder in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) (http://www.dsm5.org/Pages/Default.aspx). Prevalence of premenstrual dysphoric disorder varies from 1.3% to 8% [18, 19]. Symptoms of this disorder significantly impair daily functioning [20, 21], and its etiology is unclear [16, 18, 20]. It raises the question whether *T. gondii* infection plays a role in this disorder as it does in other psychiatric disorders. To our knowledge, the link of *T. gondii* infection and premenstrual dysphoric disorder has not been studied. Therefore, we sought to determine the association of *T. gondii* infection with clinical characteristics of premenstrual dysphoric disorder in a sample of women in Durango City, Mexico.

Materials and Methods

Study design and population studied

Through a cross-sectional study, we examined 151 women with premenstrual dysphoric disorder. Women studied were enrolled in two public primary healthcare centers: Centro de Salud #2 of the Secretary of Health (n = 78), and Clinic of Family Medicine of the Institute of Security and Social Services of State Workers (n = 73) in the northern Mexican city of Durango. Participants were examined from February to April 2016. Inclusion criteria for enrollment were women suffering from premenstrual dysphoric disorder, aged 30 - 40 years old, and who accepted to participate in the study. Diagnosis of premenstrual dysphoric disorder was made according to the DSM-5 criteria [22]. Occupation, civil status, and socioeconomic level of women were not restrictive criteria for enrollment. Pregnant women were not included in the study. Mean age in women examined was 35.52 ± 3.59.

Clinical data of the women studied

Signs and symptoms of women were obtained with the aid of a questionnaire. In total, 59 clinical characteristics of women were recorded including presence of irregular periods, severity of menstruation, vaginal infections, painful periods, fatigue, memory lapses, difficulty concentrating, confusion, judgment problems, suffering from mental illness, mood changes, low self-esteem, depression, guilty feeling, increase of fears, panic attacks, anxiety, tension, nervousness, irritability, aggressiveness, lack of interest in daily activities, lack of interest in social relations, out of control feeling or overwhelmed, reduced tolerance to noises and lights, dizziness, headache, migraine, allergy, breast pain, arterial hypertension, bouts of rapid heartbeat, decrease in muscle power, joint pain, low back pain, muscle tension, clumsiness, tingling extremities, electric shock sensation, bruises, edema in ankles, hands or feet, decreased libido, increased libido, dyspareunia, abdominal bloating, gas, abdominal pain, constipation, diarrhea, nauseas, abdominal inflammation, appetite disturbance, desire to eat certain food or eat a lot, weight gain, respiratory problems, presence of acne, presence of herpes labialis, sleep problems, thyroid disease, and obesity.

Detection of anti-*T. gondii* IgG and IgM antibodies

A serum sample from each woman was obtained. Serum samples were stored at -20 °C until analyzed. Anti-*T. gondii* IgG antibodies were detected in serum samples with the commercially available enzyme immunoassay (EIA) kit "*Toxoplasma* IgG" (Diagnostic Automation/Cortez Diagnostics Inc., Woodland Hills, CA, USA). This kit was also used to quantitate the IgG levels. Anti-*T. gondii* IgG antibody levels were expressed as International Units (IU)/mL. A cut off of ≥ 8 IU/mL of IgG was used for seropositivity. Sera reactive for anti-*T. gondii* IgG antibodies were further analyzed for anti-*T. gondii* IgM antibodies by the commercially available EIA "*Toxoplasma* IgM" kit (Diagnostic Automation/Cortez Diagnostics Inc.). All EIAs were performed following the instructions of the manufacturer.

Detection of T. gondii DNA

Whole blood of women with anti-*T. gondii* IgG antibodies was analyzed to detect *T. gondii* DNA by nested-polymerase chain reaction (PCR). DNA of whole blood was extracted following a protocol describe elsewhere (http://www.protocol-online.org/prot/Protocols/Rapid-Extraction-of-High-Quality-DNA-from-Whole-Blood-Stored-at-4-C-for-Long-Period-4175.html). Primers directed against the B1 gene of *T. gondii* and a PCR protocol previously described [23] were used. Sensitivity and specificity of this test were previously examined [24]. Amplification products were analyzed by electrophoresis using 2% agarose gels, stained with ethidium bromide, and visualized using ultraviolet illumination.

Statistical analysis

Results were analyzed with the aid of the software SPSS 15.0 (SPSS Inc. Chicago, IL), Microsoft Excel, and Epi Info 7. For calculation of the sample size, we used: 1) a reference seroprevalence of 6.1% [25] as the expected frequency for the factor under study, 2) 100,000 as the population size from which the sample was selected, 3) a 4.0% of confidence limits, and 4) a 95% confidence level. The result of the sample size calculation was 137 subjects. The association of *T. gondii* infection and the clinical characteristics of women was assessed with the Pearson's Chi-squared test or the two-tailed Fisher's exact test (when values were five or less). In the multivariate analysis, we included only variables with a P value ≤ 0.20 obtained

Table 1. Results of Bivariate Analysis of a Selection of Clinical Data and Seropositivity to *T. gondii*

Characteristics	No. of women tested	Prevalence of *T. gondii* infection		P value
		No.	%	
Irregular periods				
Yes	100	9	9	0.16
No	50	1	2	
Sleep problems				
Yes	85	4	4.7	0.33
No	66	6	9.1	
Difficulty concentrating				
Yes	109	4	3.7	0.02
No	41	6	14.6	
Judgment problems				
Yes	59	2	3.4	0.31
No	91	8	8.8	
Mood changes				
Yes	145	9	6.2	0.29
No	5	1	20	
Low self-esteem				
Yes	114	5	4.4	0.06
No	37	5	13.5	
Guilty feeling				
Yes	80	3	3.8	0.19
No	71	7	9.9	
Increase of fears				
Yes	83	3	3.6	0.11
No	68	7	10.3	
Anxiety				
Yes	103	5	4.9	0.29
No	48	5	10.4	
Tension				
Yes	111	6	5.4	0.45
No	40	4	10	
Nervousness				
Yes	119	7	5.9	0.44
No	32	3	9.4	
Irritability				
Yes	124	7	5.6	0.38
No	27	3	11.1	
Lack of interest in social relations				
Yes	92	8	8.7	0.31
No	59	2	3.4	
Out of control feeling or overwhelmed				
Yes	86	8	9.3	0.18
No	65	2	3.1	

Table 1. Results of Bivariate Analysis of a Selection of Clinical Data and Seropositivity to *T. gondii* - *(continued)*

Characteristics	No. of women tested	Prevalence of *T. gondii* infection		P value
		No.	%	
Reduced tolerance to noises and lights				
Yes	89	4	4.5	0.31
No	62	6	9.7	
Joint pain				
Yes	96	8	8.3	0.32
No	55	2	3.6	
Low back pain				
Yes	117	9	7.7	0.45
No	34	1	2.9	
Tingling extremities				
Yes	96	4	4.2	0.17
No	55	6	10.9	
Decreased libido				
Yes	81	7	8.6	0.34
No	68	3	4.4	
Dyspareunia				
Yes	42	1	2.4	0.28
No	108	9	8.3	
Presence of acne				
Yes	85	4	4.7	0.33
No	65	6	9.2	

*Sum may not add up to 151 because of missing values.

in the bivariate analysis. We calculated odds ratio (OR) and 95% confidence interval (CI) using logistic regression with the Enter method. A P value less than 0.05 was considered statistically significant.

Ethical aspects

This study was approved by the Ethics Committees of the General Hospital of the Secretary of Health, and the Institute of Security and Social Services of State Workers in Durango City, Mexico. Participation of women was voluntary. The purpose and procedures of this study were explained to all participants, and a written informed consent was obtained from all of them.

Results

Anti-*T. gondii* IgG antibodies were found in 10 (6.6%) of the 151 women with premenstrual dysphoric disorder studied. Of the 10 anti-*T. gondii* IgG positive women, five (50.0%) had IgG levels > 150 IU/mL, two (20.0%) between 100 and 150 IU/mL, and three (30.0%) between 8 and 99 IU/mL. Anti-*T. gondii* IgM antibodies were found in four (40.0%) of the 10 IgG seropositive women. DNA of *T. gondii* was detected in one (10.0%) of the 10 women with IgG antibodies against *T. gondii*. IgG levels in this women were 146 IU/mL.

Women seropositive to anti-*T. gondii* IgG antibodies showed from 13 to 40 (mean: 25.8 ± 7.58) signs or symptoms, whereas seronegative women (n = 141) had from 7 to 48 (mean: 29.22 ± 9.13) signs or symptoms. Mean number of clinical characteristics in seropositive women was similar to that found in seronegative women (P = 0.25).

Concerning clinical characteristics, bivariate analysis showed seven variables potentially ($P \leq 0.20$) associated with IgG seropositivity to *T. gondii*: irregular periods, difficulty concentrating, low self-esteem, guilty feeling, increase of fears, out of control feeling or overwhelmed, and tingling extremities. Other clinical characteristics studied showed P values higher than 0.20 by bivariate analysis. Results of bivariate analysis of a selection of clinical characteristics of women and IgG seropositivity to *T. gondii* are shown in Table 1. Further analysis by logistic regression of variables with $P \leq 0.20$ obtained by bivariate analysis showed that seropositivity to *T. gondii* was negatively associated with difficulty concentrating (OR: 0.18; 95% CI: 0.03 - 0.91; P = 0.03), and positively associated with out of control feeling or overwhelmed (OR: 9.00; 95% CI: 1.32 - 62.00; P = 0.02) (Table 2).

Bivariate analysis showed that the prevalence of high (> 150 IU/mL) IgG levels to *T. gondii* was significantly (P =

Table 2. Results of the Multivariate Analysis of Clinical Characteristics and IgG Seropositivity to *T. gondii*

Characteristics	Odds ratio	95% confidence interval	P value
Irregular periods	7.91	0.75 - 82.5	0.08
Difficulty concentrating	0.18	0.03 - 0.91	0.03
Low self-esteem	0.48	0.08 - 2.57	0.39
Guilty feeling	0.45	0.08 - 2.46	0.36
Increase of fears	0.58	0.11 - 3.10	0.53
Out of control feeling or overwhelmed	9.00	1.32 - 62.0	0.02
Tingling extremities	0.40	0.08 - 1.86	0.24

0.02) lower in women with difficulty concentrating (1/109: 0.9%) than in those without this clinical feature (4/41: 9.8%). Whereas prevalence of high IgG levels was higher, but not statistically significant (P = 0.07), in women suffering from out of control feeling or overwhelmed (5/86: 5.8%) than in those without this clinical characteristic (0/65: 0%). Concerning the association of clinical data and seropositivity to both anti-*T. gondii* IgG and IgM antibodies, bivariate analysis showed no significant associations. DNA of *T. gondii* was found in only one woman who was seropositive to anti-*T. gondii* IgG antibodies. Due to a limited number of cases with high IgG levels, seropositivity to IgM, and positivity to *T. gondii* DNA, no further regression analysis of the association of these laboratory results and the clinical variables was performed.

Discussion

Premenstrual dysphoric disorder is a clinical entity of unclear pathogenesis [18]. This psychiatric disorder is currently affecting up to 8% of women at reproductive age and focuses on psychological symptoms, whereas physical symptoms prevail in premenstrual syndrome [21]. Infection with *T. gondii* leads to a wide parasite spread in the host from the intestine to many organs in the body including the brain [26]. Infection with *T. gondii* has been associated with psychiatric disorders in general [27, 28], and it has been linked to changes in behavior [29, 30]. To our knowledge, the association between *T. gondii* infection and signs and symptoms of premenstrual dysphoric disorder was not assessed previously. Therefore, the present work aimed to determine whether infection with *T. gondii* was associated with clinical characteristics of premenstrual dysphoric disorder in a sample of women in the northern Mexican city of Durango. We observed that women with *T. gondii* IgG antibodies had a similar mean number of signs or symptoms to seronegative women. Nevertheless, multivariate analysis showed that seropositivity to *T. gondii* is associated with specific clinical characteristics of premenstrual dysphoric disorder in particular. Interestingly, IgG seropositivity to *T. gondii* was associated with the clinical manifestation of out of control feeling or overwhelmed. It is not clear why infection with *T. gondii* was associated with this psychological symptom in women with premenstrual dysphoric disorder. We did not find any report in the medical literature about the association of *T. gondii* and the clinical manifestation of out of control feeling or overwhelmed. The subjective sense of being overwhelmed or out of control has been recognized as a diagnostic symptom of premenstrual dysphoric disorder for about 20 years [31]. In a retrospective study of Brazilian college students, researchers found that out of control feeling or overwhelmed were major symptoms of premenstrual dysphoric disorder [32]. Brazil has a high (64.9%) prevalence of *T. gondii* infection among women of childbearing age [33], and our finding raises the question whether *T. gondii* might be linked to these symptoms in Brazilian women with premenstrual dysphoric disorder. In a study in Casablanca, Morocco, researchers found that the subjective sense of being overwhelmed or out of control was present in 55.7% of women with premenstrual dysphoric disorder studied [34]. We may hypothesize that *T. gondii* in brain may lead to psychological symptoms as out of control feeling or overwhelmed in women suffering from premenstrual dysphoric disorder. Infection with *T. gondii* may cause changes in neurotransmitters, i.e., dopamine and serotonin, that could lead to mood and behavioral changes [11, 35]. On the other hand, in the present study, infection with *T. gondii* was negatively associated with difficulty concentrating. This finding suggests a protective effect of *T. gondii* on this clinical characteristic. Nevertheless, this finding may also suggest that *T. gondii* was not an important factor for this symptom in women with premenstrual dysphoric disorder. Difficulty concentrating has been linked to *T. gondii* infection in immunocompetent adult patients suffering from acute toxoplasmic lymphadenitis [10]. Difference in results among the studies might be explained by difference in the characteristics of the patients studied including clinical diagnosis of patients, duration of infection (acute vs. chronic), and gender.

This study has limitations including small sample size of women with premenstrual dysphoric disorder and a low frequency of anti-*T. gondii* IgG, IgM and PCR positivity. In addition, criteria for diagnosis of this disorder have been recently described, and it is unclear whether this disorder requires further characterization. For the above reasons results of the present study should be interpreted with care.

Conclusions

Results of this first study on the association of *T. gondii* infection and clinical characteristics of premenstrual dysphoric disorder suggest that this infection might be linked to some

symptoms of this disorder. We report for the first time the association of *T. gondii* infection and out of control feeling or overwhelmed. Results warrant for further research on the role of *T. gondii* in premenstrual dysphoric disorder.

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Competing Interests

The authors declare that no competing interests exist.

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Toxoplasma gondii exposure and Parkinson's disease: a case–control study

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ABSTRACT

Objectives: To determine the association between *Toxoplasma gondii* infection and Parkinson's disease and to investigate whether *T. gondii* seropositivity is associated with the general characteristics of patients with Parkinson's disease.

Design: Case–control study.

Setting: Cases and controls were enrolled in Durango City, Mexico.

Participants: 65 patients with Parkinson's disease and 195 age- and gender-matched control subjects without Parkinson's disease.

Primary and secondary outcome measures: Serum samples of participants were analysed for anti-*T. gondii* IgG and IgM antibodies by commercially available enzyme-linked immunoassays. Prevalence of *T. gondii* DNA was determined in seropositive subjects using PCR. The association between clinical data and infection was examined by bivariate analysis.

Results: Anti-*T. gondii* IgG antibodies were found in 6/65 cases (9.2%) and in 21/195 controls (10.8%) (OR 0.84; 95% CI 0.32 to 2.18; p=0.81). The frequency of high (>150 IU/mL) antibody levels was similar among cases and controls (p=0.34). None of the anti-*T. gondii* IgG positive cases and four of the anti-*T. gondii* IgG positive controls had anti-*T. gondii* IgM antibodies (p=0.54). The prevalence of *T. gondii* DNA was comparable in seropositive cases and controls (16.7% and 25%, respectively; p=1.0). Seroprevalence of *T. gondii* infection was associated with a young age onset of disease (p=0.03), high Unified Parkinson Disease Rating Scale scores (p=0.04) and depression (p=0.02). Seropositivity to *T. gondii* infection was lower in patients treated with pramipexole than in patients without this treatment (p=0.01). However, none of the associations remained significant after Bonferroni correction.

Conclusions: The results do not support an association between *T. gondii* infection and Parkinson's disease. However, *T. gondii* infection might have an influence on certain symptoms of Parkinson's disease. Further research to elucidate the role of *T. gondii* exposure on Parkinson's disease is warranted.

Strengths and limitations of this study

- This study provides evidence for a better understanding on the association of *Toxoplasma gondii* infection and Parkinson's disease.
- This is the first study that adds molecular detection of *T. gondii* to assess its link with Parkinson's disease.
- Matching by age and sex was performed.
- This study provides clinical characteristics of Parkinson's disease associated with *T. gondii* infection.
- The seroprevalence of *T. gondii* infection was low.

INTRODUCTION

Toxoplasma gondii (*T. gondii*) is an Apicomplexan parasite of medical importance.[1] Infections with *T. gondii* are common and occur worldwide.[2] The main routes of human infection with *T. gondii* include ingestion of water or food contaminated with parasite oocysts shed by cats and consumption of raw or undercooked meat containing parasite tissue cysts.[3] In rare cases, transmission of *T. gondii* may occur by blood transfusion or transplantation.[4] *T. gondii* spreads to a number of organs of the infected host and is able to cross biological barriers and enter into the brain, eye and placenta.[5] Primary infection with *T. gondii* during pregnancy may lead to infection of the fetus.[6] The clinical spectrum of *T. gondii* infection varies from asymptomatic to severe disease with lymphadenopathy, chorioretinitis and meningoencephalitis.[3 6 7]

Infection with *T. gondii* has been linked to a number of neuropsychiatric diseases including schizophrenia, Parkinson's disease and Alzheimer's disease, and the

neurobiological data of this link have recently been reviewed.[8] The aetiology of Parkinson's disease is largely unknown; however, progressive impairment of voluntary motor control—which is a clinical feature of this disease—is caused by a loss of midbrain substantia nigra dopamine neurons.[9] Tissue cysts of *T. gondii* may be found in all brain areas,[10] and *T. gondii* may lead to neurological damage.[11] It therefore raises the question whether infection with *T. gondii* may lead to Parkinson's disease. On the other hand, infection with *T. gondii* may increase the production of dopamine in the brain.[12] Therefore, it also raises the question whether Parkinson's disease could be negatively associated with infection with *T. gondii*. However, the potential link of *T. gondii* infection and Parkinson's disease has been poorly investigated, and conflicting results about the association of *T. gondii* exposure and Parkinson's disease have been reported. Miman *et al*[13] found a significantly higher rate of anti-*T. gondii* IgG antibodies in patients with Parkinson's disease than in controls. In contrast, Celik *et al*[14 15] found similar seropositivity rates to *T. gondii* in 50 patients with idiopathic Parkinson's disease and 50 healthy volunteers. In addition, Oskouei *et al*[16] found similar prevalences of anti-*T. gondii* IgG antibodies in 75 patients with Parkinson's disease and 75 controls. Given these conflicting results, we assessed the association of *T. gondii* infection and Parkinson's disease in a cohort of patients attending public hospitals in Durango City, Mexico. In addition, we investigated the association of *T. gondii* seropositivity and the sociodemographic and clinical characteristics of patients with Parkinson's disease.

MATERIALS AND METHODS

Patients with Parkinson's disease and controls

We performed a case–control study of 65 patients with Parkinson's disease (cases) and 195 control subjects. Diagnosis of Parkinson's disease was made using the UK Parkinson's Disease Society brain bank clinical diagnostic criteria.[17] Patients were enrolled in the departments of neurology at two public hospitals: the Hospital 'Santiago Ramón y Cajal' of the Institute of Security and Social Services for the State Workers, and the Hospital '450' of the Secretary of Health in Durango City, Mexico. Serum samples were obtained from January to December 2014. Inclusion criteria for the cases were patients with Parkinson's disease of either sex who voluntarily accepted to participate in the study. Exclusion criteria for the cases were presence of renal or liver diseases, gout, alcoholism, history of cerebrovascular disease or other neurological diseases, and use of acetylsalicylic acid or allopurinol. Cases were aged 39–95 years (mean 69.08±11.39 years) and included 30 men and 35 women. We used a convenience sampling to enrol cases. Inclusion criteria for controls were subjects from the general population of the same city without neurological disease, matched with cases by age and sex. We included three controls per case. Controls were aged 38–91 years (mean 68.56±10.08 years) and included 90 men and 105 women. There was no difference in age between cases and controls (p=0.85).

Sociodemographic and clinical data of cases

We obtained the sociodemographic and clinical data of the patients with Parkinson's disease through face-to-face neurological consultations and with the aid of a questionnaire. Since the correlation of *T. gondii* infection with clinical features of Parkinson's disease is largely unknown, we explored the association between *T. gondii* seropositivity and a number of clinical characteristics directly or indirectly associated with Parkinson's disease. Sociodemographic data obtained included age and sex. Clinical data included Hoehn and Yahr stages,[18] Unified Parkinson Disease Rating Scale scores, age at onset of Parkinson's disease, duration of disease, presence of tremor or rigidity at disease onset, most affected body side, familial history of Parkinson's disease, presence of hyposmia, syncope, paraesthesias, dementia, impairments of memory and vision, depression, anxiety, sialorrhoea, constipation, weight loss, sleep disorders, erectile dysfunction and orthostatic hypotension. In addition, information about the presence of obesity, dyslipidaemia, diabetes mellitus, arterial hypertension, smoking, diarrhoea, nausea and/or vomiting was obtained from each patient. Antiparkinsonian medication was also registered and included the use of levodopa, carbidopa, pramipexole, trihexyphenidyl, biperiden, amantadine, rasagiline, selegiline, azilect, rotigotine and bromocriptine. The occurrence of dyskinesia, urinary incontinence and motor fluctuations (ie, end-of-dose wearing-off, unpredictable off, delay on and no on) related to treatment was also recorded.

Detection of anti-*T. gondii* antibodies

Anti-*T. gondii* IgG antibodies were detected in the serum of participants using the commercially available enzyme immunoassay *Toxoplasma* IgG kit (Diagnostic Automation, Woodland Hills, California, USA). This test determines the presence and also the levels of IgG antibodies. A cut-off of 8 IU/mL of specific anti-*T. gondii* IgG antibody was used. All serum samples positive for anti-*T. gondii* IgG antibodies were further analysed for anti-*T. gondii* IgM antibodies by the commercially available enzyme immunoassay *Toxoplasma* IgM kit (Diagnostic Automation). All tests were performed following the manufacturer's instructions.

Detection of *T. gondii* DNA by PCR

Whole blood samples of cases and controls with anti-*T. gondii* IgG antibodies were further examined to detect *T. gondii* DNA by nested PCR. Whole blood extraction of DNA followed the protocol described by Iranpour and Esmailizadeh (http://www.protocol-online.org/prot/Protocols/Rapid-Extraction-of-High-Quality-DNA-from-Whole-Blood-Stored-at-4-C-for-Long-Period-4175.html). A PCR protocol with two pairs of primers directed

against the B1 gene of *T. gondii* was used, as previously described.[19] The amplified PCR products were detected using gel electrophoresis, stained with ethidium bromide and visualised under ultraviolet light.

Statistical analysis

We used the software Microsoft Excel 2010, Epi Info V.7 (Centers for Disease Control and Prevention: http://wwwn.cdc.gov/epiinfo/) and SPSS V.15.0 (SPSS, Chicago, Illinois, USA) to analyse the results. For calculation of the sample size we used a 95% confidence level, power of 80%, 1:3 proportion of cases and controls and a reference seroprevalence of 12.0%[20] as the expected frequency of exposure in controls. The result of the sample size calculation was 60 cases and 179 controls. To avoid bias, we excluded subjects with missing clinical data. Age values among the groups were compared with the paired Student's t-test. The Fisher exact test was used to evaluate the association between seropositivity to *T. gondii* and the characteristics of the patients. ORs and 95% CIs were calculated and a p value <0.05 was considered statistically significant. Bonferroni correction was applied for adjustment of multiple testing.

RESULTS

Anti-*T. gondii* IgG antibodies were found in 6/65 cases (9.2%) and in 21/195 controls (10.8%) (OR 0.84; 95% CI 0.32 to 2.18; p=0.81). Of the six anti-*T. gondii* IgG positive cases, five (83.3%) had anti-*T. gondii* IgG antibody levels >150 IU/mL and one (16.7%) 12 IU/mL. In contrast, of the 21 anti-*T. gondii* IgG positive controls, 11 (52.4%) had anti-*T. gondii* IgG antibody levels >150 IU/mL, one (4.8%) between 100 to 150 IU/mL and 9 (42.8%) between 8 and 99 IU/mL. The frequency of high (>150 IU/mL) antibody levels was similar among cases and controls (p=0.34). None of the six anti-*T. gondii* IgG positive cases had anti-*T. gondii* IgM antibodies whereas four (19.0%) of the 21 anti-*T. gondii* IgG positive controls had anti-*T. gondii* IgM antibodies. There was no difference in the rate of IgM seropositivity among cases and controls (p=0.54). Anti-*T. gondii* IgG antibodies were detected in four (11.4%) of 35 female cases and in seven (6.7%) of 105 female controls (OR 1.80; 95% CI 0.49 to 6.58; p=0.46), whereas anti-*T. gondii* IgG antibodies were detected in two (6.7%) of 30 male cases and in 14 (15.6%) of 90 male controls (OR 0.38; 95% CI 0.08 to 1.81; p=0.35). The frequency of high (>150 IU/mL) anti-*T. gondii* IgG antibody levels was similar in male and female cases (2/30 (6.7%) and 3/35 (8.6%), respectively, p=1.00). Seroprevalence of *T. gondii* infection was similar among cases and controls of several age groups (table 1). One (16.7%) of the six cases seropositive to *T. gondii* IgG antibodies was positive for *T. gondii* DNA by PCR. We were able to test 20 of 21 controls seropositive to *T. gondii* IgG antibodies. Five (25%) of these 20 controls were positive for *T. gondii* DNA by PCR. The prevalence of *T. gondii* DNA was similar in cases and controls (p=1.0).

With respect to clinical characteristics of patients, seroprevalence of *T. gondii* infection was higher in patients with an onset of Parkinson's disease at a young age (≤40 years) than in those with a disease onset at older ages (p=0.03). Table 2 shows a selection of clinical characteristics of patients with Parkinson's disease and their correlation with *T. gondii* seropositivity. Seroprevalence of infection with *T. gondii* was also higher in patients with higher Unified Parkinson Disease Rating Scale scores (88–136) than in those with lower scores (p=0.04). Seropositivity to *T. gondii* was observed in six (17.1%) of 35 patients suffering from depression but in none of 30 patients without depression (p=0.02). Other clinical characteristics of patients including Hoehn and Yahr stages, duration of disease, presence of tremor or rigidity at disease onset, most affected body side, familial history of Parkinson's disease, presence of hyposmia, syncope, paraesthesias, dementia, impairments of memory and vision, anxiety, sialorrhoea, constipation, weight loss, sleep disorders, erectile dysfunction, and orthostatic hypotension did not show an association with *T. gondii* seropositivity. In addition, *T. gondii* exposure was not associated with the presence of obesity, dyslipidaemia, diabetes mellitus, arterial hypertension, smoking, diarrhoea, nausea and/or vomiting in the patients. Seropositivity to *T. gondii* infection was significantly (p=0.01) lower in patients receiving pramipexole than in patients not treated with this drug (table 2). Seroprevalence of infection was similar in patients regardless of the use of other antiparkinsonian medications including levodopa, carbidopa, trihexyphenidyl, biperiden, amantadine, rasagiline, selegiline, azilect, rotigotine and bromocriptine. The presence of

Table 1 Comparison of IgG seropositivity rates in cases and controls according to age groups

	Cases			Controls			
	Subjects tested	Seropositive		Subjects tested	Seropositive		
	N	N	%	N	N	%	p Value
Age groups							
≤40 years	2	1	50	6	0	0	0.25
41–60 years	12	1	8.3	22	1	4.5	1.00
61–80 years	41	4	9.8	144	17	11.8	1.00
>80 years	10	0	0	23	3	13	0.53

Table 2 Bivariate analysis of clinical data and infection with *Toxoplasma gondii* in patients with Parkinson's disease

Characteristic	Subjects tested N	Prevalence of *T. gondii* infection N	%	p Value
Age at Parkinson onset				
≤40 years	4	2	50	0.03
>40 years	61	4	6.6	
Duration of disease				
≤10 years	57	5	8.8	0.56
>10 years	8	1	12.5	
Tremorigenic type				
Yes	49	5	10.2	1.00
No	16	1	6.3	
Rigid type				
Yes	25	3	12	0.66
No	40	3	7.5	
Hoehn and Yahr stages				
0	5	0	0	0.59
1	17	3	17.6	
2	14	1	7.1	
3	20	1	5	
4	5	1	20	
5	4	0	0	
Unified Parkinson disease rating scores				
0–87	55	3	5.5	0.04
88–136	10	3	30	
Constipation				
Yes	29	4	13.8	0.39
No	36	2	5.6	
Syncope				
Yes	6	1	16.7	0.45
No	59	5	8.5	
Paraesthesias				
Yes	12	3	25	0.07
No	53	3	5.7	
Weight loss				
Yes	27	4	14.8	0.22
No	38	2	5.3	
Dementia				
Yes	23	3	13	0.65
No	42	3	7.1	
Depression				
Yes	35	6	17.1	0.02
No	30	0	0	
Anxiety				
Yes	30	4	13.3	0.40
No	35	2	5.7	
Vision impairment				
Yes	22	3	13.6	0.39
No	43	3	7	
Dyskinesia				
Yes	21	3	14.3	0.37
No	44	3	6.8	
Use of pramipexole				
Yes	43	1	2.3	0.01
No	22	5	22.7	

dyskinesia, urinary incontinence and motor fluctuations (end-of-dose wearing-off, unpredictable off, delay on and no on) did not correlate with *T. gondii* infection. None of the associations between clinical data and *T. gondii* seropositivity remained significant after Bonferroni correction.

DISCUSSION

T. gondii is an intracellular parasite and can persist in neurons, modifying their function and structure.[21] Cysts of *T. gondii* can be found throughout the brain,[10] and this parasite alters dopamine metabolism.[21] Thus, it raises the question whether infection with *T. gondii* has any link with a dopamine-related neurological disease. There is controversy concerning the association of *T. gondii* infection and Parkinson's disease. The number of reports about this association is very small. We therefore sought to determine the association between *T. gondii* seropositivity and patients with Parkinson's disease in the northern Mexican city of Durango. This age- and gender-matched case–control seroprevalence study showed similar frequencies of *T. gondii* infection in cases and controls. Similarly, we did not find differences in the frequency of high levels of anti-*T. gondii* IgG antibodies, IgM seropositivity rates and prevalence of *T. gondii* DNA among cases and controls. The 9.2% seroprevalence found in patients with Parkinson's disease is comparable to the 12% seroprevalence of *T. gondii* infection reported in elderly people[20] and 13.3% in patients with liver disease[22] in the same Durango City. In contrast, the seroprevalence found in patients with Parkinson's disease is lower than seroprevalences reported in other population groups in Durango City including 15.4% in female sex workers,[23] 20% in schizophrenic patients[24] and 21.1% in inmates[25] and waste pickers.[26] Therefore, the results of our study do not support an association between *T. gondii* infection and Parkinson's disease. The lack of association between *T. gondii* infection and the presence of Parkinson's disease is consistent with similar results reported by Celik *et al*[14 15] and Oskouei *et al*.[16]

In contrast, our results conflict with those reported by Miman *et al*[13] who found a significantly higher seroprevalence of anti-*T. gondii* IgG antibodies in patients with Parkinson's disease than in controls. Other studies have also linked toxoplasmosis with Parkinson's disease. For instance, in 1992 Noël *et al*[27] reported hemichorea and parkinsonism in two AIDS patients with cerebral toxoplasmosis. Basal ganglia, which are involved in the control of voluntary motor movements, can be affected in cerebral toxoplasmosis, as reported in patients with AIDS,[28–30] a patient with acute myeloid leukaemia undergoing two allogenic stem cell transplantations,[31] an immunocompromised female renal transplant recipient[32] and a non-immunocompromised pregnant woman.[33] Improvement of parkinsonism in an AIDS patient with cerebral toxoplasmosis was achieved after anti-*T. gondii* and antiretroviral therapies.[34] Infection with *T. gondii* has been associated with elevated levels of dopamine within dopaminergic cells,[12] whereas an important feature of Parkinson's disease is the loss of dopamine-producing neurons.[35] However, the interaction of *T. gondii* and neurons in patients with Parkinson's disease is largely unknown. It raises the question whether dopamine production during infection with *T. gondii* is too low to compensate for the deficit of dopamine and to induce a clinical improvement in patients with Parkinson's disease. Further research to elucidate the role of dopamine produced during *T. gondii* infection on neurons of patients with Parkinson's disease is needed.

Interestingly, the frequency of *T. gondii* infection was higher in patients with onset of Parkinson's disease at a young age than in those with a disease onset at older ages. It is not clear why *T. gondii* infection was associated with a young onset of Parkinson's disease. This young onset of disease is less common than middle and late onsets, and patients with young onset have a long survival and suffer from depression more frequently than patients with older onset of disease.[36] Remarkably, we found that seropositivity to *T. gondii* was associated with depression in the patients with Parkinson's disease studied. To the best of our knowledge, this is the first report of an association between *T. gondii* exposure and depression in patients with Parkinson's disease. Infection with *T. gondii* has been linked to depression in other population groups, such as women veterans[37] and pregnant women.[38] However, other studies including a meta-analysis of 50 studies of psychiatric patients and healthy controls,[39] a cross-sectional internet study on a non-clinical population of 5535 subjects[40] and the third National Health and Nutrition Survey in the USA[41] have not found a correlation between *T. gondii* infection and depression

Of note, seroprevalence of *T. gondii* infection correlated with high Unified Parkinson Disease Rating Scale scores. In a search for this association in the medical literature, no reports were found. This association suggests that *T. gondii* infection might have an influence on clinical characteristics of patients with Parkinson's disease. It is possible that *T. gondii* does not associate per se with the presence of Parkinson's disease because of the opposite relations with dopamine production—that is, *T. gondii* infection induces an increase in dopamine production whereas Parkinson's disease is related to a decrease in dopamine production. However, infection with *T. gondii* might be involved in the appearance of symptoms found in patients with Parkinson's disease such as depression. Further research on the influence of *T. gondii* infection on signs and symptoms of Parkinson's disease should be conducted.

We also observed that seropositivity to *T. gondii* infection was significantly lower in patients treated with pramipexole than in those not receiving this treatment. This finding suggests a protective effect of pramipexole for *T. gondii* infection. It is not clear why pramipexole users had a low frequency of *T. gondii* infection. No anti-*T. gondii* activity of pramipexole has been reported. Further research to elucidate the negative association of pramipexole with *T. gondii* infection is needed.

This study has limitations. The sample size was small. Further studies with larger sample sizes should be conducted. The low number of cases seropositive for

T. gondii did not allow us to perform multivariate analysis to determine the association between patient characteristics and seropositivity to *T. gondii*. In addition, the associations between clinical data and *T. gondii* seropositivity found in this study should be interpreted with care, since the statistical power of comparisons was low (<0.80) and no associations remained statistically significant after Bonferroni correction.

CONCLUSIONS

The results obtained in a cohort of patients in Durango, Mexico do not support an association between *T. gondii* infection and Parkinson's disease. However, the results suggest that *T. gondii* infection might influence the symptoms of Parkinson's disease. Further research to elucidate the role of *T. gondii* exposure on the clinical characteristics of Parkinson's disease is therefore needed.

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Contributors CA-E designed the study protocol, performed the laboratory tests and data analysis and wrote the manuscript. EMM-H, JMS-P, LAR-C and AAS-C obtained the blood samples and clinical dat, and performed the data analysis. JH-T, OA-C, LFS-A, FXC-J and OL performed the data analysis and wrote the manuscript. All authors read and approved the final version of the manuscript.

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ORIGINAL ARTICLE

Lack of Serological and Molecular Association between *Toxoplasma Gondii* Exposure and Obesity: A Case-Control Study

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ABSTRACT

The association between *T. gondii* infection and obesity has been scantly studied. Through an age-, and gender-matched case-control study, we determined the association of *T. gondii* infection and obesity using serological and molecular methods. Cases included 203 persons with obesity, and controls included 203 persons without obesity. Participants were tested for the presence of anti-*Toxoplasma* IgG antibodies using an enzyme-linked immunoassay (EIA). IgG seropositive individuals were further tested for the presence of anti-*T. gondii* IgM antibodies using an EIA, and *T. gondii* DNA by polymerase chain reaction (PCR). Anti-*T. gondii* IgG antibodies were found in 16 (7.9%) of the 203 cases and in 18 (8.9%) of the 203 controls (OR=0.87; 95% CI: 0.43-1.77; *P*=0.72). One (6.3%) of the 16 anti-*T. gondii* IgG seropositive cases and 6 (33.3%) of the 18 anti-*T. gondii* IgG seropositive controls were positive for IgM (*P*=0.09). Mean body mass index (35.5 ± 4.5) in *T. gondii* seropositive cases was similar (*P*=0.57) to that (36.1 ± 4.5) found in *T. gondii* seronegative cases. Stratification by obesity classes (I, II, and III) did not reveal differences (*P*>0.05) in seroprevalences (7.8%, 7.9%, and 8.1%, respectively) or high (>150 IU/ml) IgG antibody levels (3.3%, 3.9%, and 2.7%, respectively). PCR was positive in 5 (31.3%) of 16 cases, and in 5 (27.8%) of 18 controls examined (*P*=1.0). We found no serological or molecular evidence of an association between *T. gondii* infection and obesity in people attending a public health center in the northern Mexican city of Durango. (*Int J Biomedi Sci* 2017; 13 (2): 74-78)

Keywords: *Toxoplasma gondii*; seroprevalence; obesity; case-control study

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INTRODUCTION

The coccidian parasite *Toxoplasma gondii* (*T. gondii*) is a common pathogen with worldwide distribution (1). This parasite is usually transmitted to humans by ingestion of food or water contaminated with oocysts shed by cats, and ingestion of raw or undercooked meat containing tissue cysts (2, 3). In addition, primary infection with *T. gondii* in pregnant women may lead to vertical transmission with risk for congenital disease (4). Infection with *T. gondii* is usually asymptomatic, however, this infection may lead to disease of the central nervous system, eyes and lymph nodes (5, 6). In immunocompromised individuals, a reactivation of *T. gondii* infection may result in a severe and life-threatening disease with involvement of the central nervous system (2, 7). Infection with *T. gondii* has been associated with changes in behavior in humans and animals (8, 9). An increase in dopamine production induced by *T. gondii* may contribute to behavioral changes (8). Several psychiatric disorders have been linked to infection with *T. gondii* including schizophrenia (10), bipolar disorder, and obsessive-compulsive disorder (11).

Obesity is a major health problem, and its prevalence is high in many parts of the world (12). In Mexico, more than 50% of adults have overweigh and obesity (13). Obesity and overweight have been linked to important causes of mortality in Mexico including coronary heart disease, type-2 diabetes mellitus, cancer, and stroke (13). Infection with *T. gondii* might be associated with obesity because this infection is usually acquired by food. Obese people may eat in a higher quantity than non-obese people; and therefore, this increase in eating might increase the risk of consuming food likely contaminated with *T. gondii*. It is possible that consumption of a double portion of meat (a well-known source of *T. gondii*) might increase two-fold the risk for acquiring infection. It is also possible that drinking untreated water or consuming unwashed raw vegetables or fruits in high quantities might also increase the risk for *T. gondii* infection. The association of *T. gondii* infection and obesity has been scantly studied. Reeves and coworkers (14) found an association between positive serology to *T. gondii* and obesity in psychiatrically healthy adults. Rubicz and coworkers (15) found a 9% seroprevalence of *T. gondii* infection in Mexican Americans from San Antonio, Texas that suffered from high rates of obesity and type-2 diabetes. In contrast, in a multinational epidemiological study of individuals from Iceland, Sweden and Estonia, no association of anti-*T. gondii* IgG antibodies and body mass index was found (16). However, in a recent study in Germany a body mass index ≥30 was an independent risk factor for IgG seropositivity to *T. gondii* (17). The present study therefore aimed to determine whether *T. gondii* infection is associated with obesity in adults attending a public clinic of family medicine in Durango City, Mexico. Determining this association may help for an optimal planning of preventive measures against *T. gondii* infection.

METHODS

Selection and description of participants

Through an age- and gender-matched case control study design, we studied 203 individuals with obesity and 203 individuals without obesity attended in a public clinic of familiar medicine in Durango City, Mexico. This study was performed from June 2015 to August 2016. Inclusion criteria for enrollment of cases were: 1) individuals with obesity attending a public primary health care center (Clinic of Family Medicine, Institute of Security and Social Services of State Workers) in Durango City, Mexico; 2) aged 18 years and older; and 3) who accepted to participate in the study. Socioeconomic status and occupation were not restrictive criteria for enrollment.

Obesity was defined as a body mass index ≥30; and classified in class I, class II, and class III when body mass indexes were 30-34.9, 35.0-39.9, and ≥40.0, respectively (13). Control subjects were matched with cases for age and gender. Cases included 42 (20.7%) males and 161 (79.3%) females, and their mean age was 51.4 ± 11.6 (range 22-83) years old. Controls were randomly selected. Inclusion criteria for enrollment of controls subjects were: 1) individuals without obesity attending the same public primary health care center where cases were selected; 2) aged 18 years and older; and 3) who accepted to participate in the study. Controls included 42 (20.7%) males and 161 (79.3%) females. Mean age in control subjects were 51.5 ± 11.5 (range 20-80) years old. No statistically significant difference (P=0.89) in age between cases and controls was found.

Technical information

Sera from cases and controls were obtained and kept frozen at -20°C until analyzed. Anti-*T. gondii* IgG antibodies were detected in sera using the commercially available enzyme immunoassay (EIA) kit "*Toxoplasma* IgG" (Diagnostic Automation/Cortez Diagnostics Inc., Woodland Hills, CA, USA). Anti-*T. gondii* IgG antibody levels were expressed as International Units (IU)/ml. We used

a cut-off of 8 IU/ml for seropositivity. All serum samples positive for anti-*T. gondii* IgG antibodies were further analyzed for anti-*T. gondii* IgM antibodies using the commercially available EIA "*Toxoplasma* IgM" kit (Diagnostic Automation/Cortez Diagnostics Inc.). Both IgG and IgM EIAs were performed following the manufacturer's instructions.

Cases and controls seropositive for *Toxoplasma*-specific IgG antibodies by EIA were further analyzed to detect DNA of *T. gondii* by nested-polymerase chain reaction (PCR). DNA was extracted from whole blood samples of cases and controls according to a protocol described by Iranpour and Esmailizadeh [http://www.protocol-online.org/prot/Protocols/Rapid-Extraction-of-High-Quality-DNA-from-Whole-Blood-Stored-at-4-C-for-Long-Period-4175.html]. PCR amplification was performed following the PCR protocol described by Roth *et al* (18). Primers directed against the B1 gene of *T. gondii* were used. PCR amplified material was analyzed by agarose gel electrophoresis, stained with ethidium bromide, and visualized by ultraviolet illumination.

Statistics

Data was analyzed using the software Epi Info 7 and SPSS 15.0 (SPSS Inc. Chicago, Illinois). We calculated the sample size using the following values: a 95% confidence level, a power of 80%, a 1:1 proportion of cases and controls, and a reference seroprevalence of 6.1% (19) as the expected frequency of exposure in controls, and an odds ratio of 2.8. Thus, a sample size of 195 cases and 195 controls was obtained. Age among cases and controls was compared with the student's *t* test. The association of *T. gondii* infection and obesity was analyzed with the two-tailed Pearson's chi-squared test. We calculated the odds ratio (OR) and 95% confidence interval (CI), and statistical significance was set at a P value < 0.05.

Ethics aspects

The ethics committee of the Institute of Security and Social Services of State Workers in Durango City, Mexico approved this study. Participation in the study was voluntary, and a written informed consent was obtained from each participant.

RESULTS

Anti-*T. gondii* IgG antibodies were found in 16 (7.9%) of the 203 cases and in 18 (8.9%) of the 203 controls. The seroprevalence of *T. gondii* infection in cases was similar to the one in controls (OR=0.87; 95% CI: 0.43-1.77; P=0.72). Of the 16 anti-*T. gondii* IgG positive cases, 7 (43.8%) had IgG levels higher than 150 IU/ml, one (6.3%) between 100-150 IU/ml, and 8 (50.0%) between 8 to 99 IU/ml. Whereas, of the 18 anti-*T. gondii* IgG positive controls, 13 (72.2%) had IgG levels higher than 150 IU/ml, and 5 (27.8%) between 8 to 99 IU/ml. The frequency of high (>150 IU/ml) anti-*T. gondii* IgG levels in cases was similar to the one in controls (OR=0.29; 95% CI: 0.07-1.24; P=0.18). One (6.3%) of the 16 anti-*T. gondii* IgG seropositive cases was positive to anti-*T. gondii* IgM antibodies by EIA. In contrast, 6 (33.3%) of the 18 anti-*T. gondii* IgG seropositive controls were positive to IgM by EIA. No difference in the frequencies of anti-*T. gondii* IgM antibodies among cases and controls was found (P=0.09).

Mean body mass index in *T. gondii* seropositive cases (35.5 ± 4.5) was similar (P=0.57) to that (36.1 ± 4.5) found in *T. gondii* seronegative cases. Stratification by obesity classes I, II, and III did not show differences (P>0.05) in seroprevalences (7.8%, 7.9%, and 8.1%, respectively) or frequency of high IgG antibody levels (3.3%, 3.9%, and 2.7%, respectively).

With respect to detection of *T. gondii* DNA in whole blood of anti-*T. gondii* IgG positive participants, PCR was positive in 5 (31.3%) of 16 cases and in 5 (27.8%) of 18 controls examined. No statistically significant difference in the frequencies of *T. gondii* DNA positivity among cases and controls was found (P=1.0). Stratification by age and gender groups did not show differences (P>0.05) in seroprevalences among cases and controls (Table 1). *T. gondii* DNA was found in three cases with >150 IU/ml of IgG antibodies and in two cases with <100 IU/ml of IgG antibodies. All 5 cases with *T. gondii* DNA were negative to anti-*T. gondii* IgM antibodies. *T. gondii* DNA was found in three cases with obesity class I, in one case with obesity class II, and in one case with obesity class III.

DISCUSSION

Very little is known about the association of *T. gondii* infection and obesity. Results of a few studies about this association have shown conflicting results (14-17). Positive association between seroprevalence of *T. gondii* infection and obesity has been found in adults in Germany (14, 17). In contrast, a low (9%) seroprevalence of *T. gondii* infection in Mexican Americans from San Antonio, Texas that suffered from high rates of obesity and type-2 diabetes was found (15). In addition, no association of seroprevalence of *T. gondii* infection and body mass index was

Table 1. Correlation of *T. gondii* seropositivity and demographic variables in cases and controls

Variable	Cases			Controls			*P.* value
	No. of subjects tested	Seropositive to T. gondii		No. of subjects tested	Seropositive to T. gondii		
		No.	%		No.	%	
Ages (years)							
30 or less	12	0	0.0	10	1	10.0	0.45
31-50	76	5	6.6	73	7	9.6	0.55
>50	115	11	9.6	120	10	8.3	0.82
Gender							
Female	161	12	7.5	161	9	5.6	0.49
Male	42	4	9.5	42	9	21.4	0.13

found in a multinational epidemiological study in Iceland, Sweden and Estonia, (16). Therefore, we sought to determine whether *T. gondii* infection is associated with obesity in adults attending a public clinic of family medicine in the northern Mexican city of Durango. For this purpose, we assessed not only the prevalence of anti-*T. gondii* IgG antibodies but also the IgG levels, anti-*T. gondii* IgM seropositivity, and detection of *T. gondii* DNA. Results of the present study indicate that anti-*T gondii* IgG and IgM seropositivity rates, IgG levels, and frequency of *T. gondii* DNA in obese people are similar to those observed in age- and gender-matched control subjects without obesity attended in the same clinic of family medicine. Therefore, our results based on serological and molecular methods do not support an association between obesity and *T. gondii* infection. Results of the present study agree with the lack of association between body mass index and *T. gondii* IgG seroprevalence found in a multinational epidemiological study of individuals from Iceland, Sweden and Estonia (16), and with the low (9%) seroprevalence of *T. gondii* infection found in Mexican Americans that suffered from high rates of obesity and type-2 diabetes reported by Rubicz and coworkers (15). In contrast, our results conflict with those reported in two German studies (14, 17). A positive serology to *T. gondii* associated with obesity in psychiatrically healthy adults in Germany was reported by Reeves and coworkers (14). Furthermore, a body mass index ≥30 was an independent risk factor for IgG seropositivity to *T. gondii* in a nationwide representative cross-sectional study in Germany (17). It is not clear why the association of *T. gondii* infection and obesity was found in populations in Germany but not in obese people in the present study. It is likely that differences in the characteristics of the populations and study designs among the studies might explain the differences in the association. In the study of Reeves and coworkers (14), the association between *T. gondii* infection and obesity was observed in subjects 60 years and older but not in subjects younger than 60 years. Stratification by age groups in our study (<30, 31-50, and >50 years) did not show an association of infection and obesity. In addition, we used an age- and gender-matched case-control study design whereas Reeves and coworkers performed adjustment by age but not by gender. The number of obese participants in the study by Reeves and coworkers was 74 (14), whereas we studied 203 obese participants. On the other hand, in the study of Wilking and coworkers (17) who reported an association of *T. gondii* seropositivity and obesity, researchers studied a large number (1,023) of obese participants, but the study design was cross-sectional, and no adjustment or stratification by age for *T. gondii* seropositivity was performed. In addition, Wilking and coworkers (17) used an automatic enzyme-linked fluorescence assay for detection of anti-*T. gondii* IgG antibodies whereas we used a manual enzyme-linked immunosorbent assay. We are not aware of a previous study about the association of *T. gondii* infection and obesity using molecular methods. However, in the present study using *T. gondii* PCR, no association between obesity and *T. gondii* DNA was found. The lack of association between obesity and *T. gondii* was unexpected since obese people may eat more than non-obese people; and therefore, an increase in eating might increase the risk of consuming food likely contaminated with *T. gondii*.

The limitations of the present study include the investigation of a relatively small cohort of obese people attending a single public health center. The socioeconomic sta-

tus of people attending the participating health center are mostly medium, and it is not clear whether the association of obesity and *T. gondii* infection might occur in people of low or high socioeconomic status.

CONCLUSIONS

We conclude that there is not serological or molecular evidence of an association between *T. gondii* infection and obesity in people attended in a public family medicine health center in the northern Mexican city of Durango. Further research to elucidate the role of *T. gondii* in obesity is needed.

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ABBREVIATIONS

CI	Confidence interval
EIA	Enzyme immunoassay
IU	International units
OR	Odds ratio
PCR	Polymerase chain reaction

CONFLICT OF INTEREST

The authors declare that no conflict of interest exists.

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Original article

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LACK OF ASSOCIATION BETWEEN CYTOMEGALOVIRUS INFECTION AND HYPERTENSIVE DISORDERS IN PREGNANCY: A CASE-CONTROL STUDY IN DURANGO, MEXICO

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It is not clear whether infection with cytomegalovirus (CMV) is associated with hypertensive disorders in pregnant women. Through a case-control study design, 146 women suffering from hypertensive disorders in pregnancy (cases) and 146 age-matched normotensive pregnant women (controls) were examined for the presence of anti-CMV IgG and IgM antibodies with enzyme-linked immunoassays. IgM seropositive samples were further assayed by enzyme-linked fluorescent assay (ELFA).

Anti-CMV IgG antibodies were found in 138 (94.5%) controls and in 136 (93.2%) cases (odds ratio [OR] = 0.78; 95% confidence interval [CI]: 0.30–2.05; $P = 0.62$). High (>18 IU/ml) levels of anti-CMV IgG antibodies were found in 37.7% of the 138 seropositive controls and in 34.6% of the 136 seropositive cases (OR = 0.87; 95% CI: 0.53–1.43; $P = 0.59$). Anti-CMV IgM antibodies were found in 1 (0.7%) of the controls but in none of the cases using ELFA ($P = 1.0$). Seropositivity to CMV was not associated with a previous preeclampsia and was similar among cases regardless their mean systolic and diastolic blood pressures, and mean arterial blood pressure.

No serological evidence of an association between CMV infection and hypertensive disorders of pregnancy was found. Further research to elucidate the role of CMV in hypertensive disorders in pregnancy should be conducted.

Keywords: cytomegalovirus, seroprevalence, preeclampsia, HELLP syndrome, eclampsia, infection, epidemiology

Introduction

Cytomegalovirus (CMV) is a DNA virus of the Herpesviridae family and is widely distributed around the world [1]. Major routes of CMV infection are person-to-person contact [2] and blood transfusion [3]. Infections with CMV are persistent [4], and their reactivations contribute for shedding of infectious virus [2, 5]. Immunocompromised individuals infected with CMV may develop a severe disease including encephalitis, pneumonia, retinitis, and hepatitis [6]. Furthermore, CMV is an important pathogen leading to congenital infections [7–9]. The clinical spectrum of congenital infections varies from asymptomatic [10] to severe disease including mental retardation, cerebral palsy, hearing loss, and neurodevelopmental delay [9, 11]. Infections with CMV may occur in placenta [12–14]. In addition, inflammation and edema in placenta induced by CMV infections have been observed in cases of preeclampsia [13, 14]. Preeclampsia and other hypertensive disorders in pregnancy are major health problems leading to maternal and perinatal morbidity and mortality [15–17]. Worldwide estimates indicate that about 8.5 mil-

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lion pregnant women suffer from preeclampsia every year [18], and 4.6% and 1.4% of all deliveries are associated with preeclampsia and eclampsia, respectively [19]. The role of CMV in hypertension disorders in pregnancy is controversial. Several serological studies have failed to demonstrate a correlation of CMV infection and hypertensive disease in pregnancy [20–22]. In contrast, several serological studies have found an association of CMV infection with preeclampsia [23–25]. Therefore, we sought to determine the association of CMV infection and hypertensive disorders in pregnant women in Durango City, Mexico.

Materials and methods

Study design and study populations

We performed a case-control study to assess the association of CMV infection with hypertensive disorders in pregnancy using serum samples from a recent *Toxoplasma gondii* study in women in Durango City, Mexico [26]. Serum samples were obtained in a public hospital (General Hospital) from the Secretary of Health from November 2011 to September 2013. Cases and controls were matched by gender, age, attending hospital, and residence place. A 1:1 ratio for matching was used.

Women suffering from hypertensive disorders in pregnancy

Inclusion criteria for the group of cases were: 1) pregnant women at their 24–42 weeks of pregnancy suffering hypertensive disorders and proteinuria attended in the Department of Gynecology and Obstetrics of the General Hospital in Durango City, Mexico; and 2) who agreed voluntary participation in the study. Hypertensive disorders during pregnancy were mild preeclampsia, severe preeclampsia, eclampsia, and HELLP syndrome. Mild preeclampsia was defined as blood pressure ≥140/90 mmHg on 2 occasions, at least 6 h apart, and proteinuria of ≥300 mg/24 h. Severe preeclampsia was considered as blood pressure ≥160/110 mmHg on 2 occasions, at least 6 h apart, and proteinuria of ≥5 g/24 h. Eclampsia was diagnosed when hypertension, proteinuria, and seizures in a patient were found. HELLP syndrome was defined as hypertension, proteinuria and presence of hemolytic anemia, elevated liver enzymes, and low platelet count. All eligible women attended in the Department of Gynecology and Obstetrics of the General Hospital during the study period were invited to participate. In total, 146 patients suffering from hypertensive disorders in pregnancy were included in the study. All of them resided in Durango City. In total, 146 cases were enrolled in the study. Of them, 27 had mild preeclampsia, 95 severe preeclampsia, 16 eclampsia, and 8 HELLP syndrome. Mean age of the cases was 23.51 ± 6.41 years (range: 15–39 years).

Control pregnant women

Inclusion criteria for the control group were: 1) pregnant women without hypertensive disorders, diabetes, or nephropathy before or during pregnancy attended in the Department of Gynecology and Obstetrics of the General Hospital in Durango City; 2) to have a normal pregnancy with systolic blood pressure <140 mmHg and diastolic blood pressure <90 mmHg; 3) patients without any underlying disease; and 4) who agreed voluntary participation in the study. Thus, 146 control women were included in this case-control study. Controls were 23.44 ± 6.17 (range: 15–39) years old, and their age was comparable to the one in cases ($P = 0.92$).

General clinical characteristics including age, number of pregnancies, cesarean sections, and deliveries, history of miscarriages and stillbirths, trimester of present pregnancy, history of preeclampsia, systolic and diastolic blood pressures, and mean arterial pressure from all participants were obtained.

Laboratory tests

Serum samples of the participants were kept frozen until analyzed. The presence of anti-CMV antibodies in serum samples was determined by commercially available enzyme immunoassays (EIA). Sera were analyzed for anti-CMV IgG antibodies by the "Cytomegalovirus IgG (CMV IgG)" kit (Diagnostic Automation Inc., Calabasas, CA, USA). In addition, all sera were tested for anti-CMV IgM antibodies by the "Cytomegalovirus IgM (CMV IgM" kit (Diagnostic Automation Inc., Calabasas, CA, USA). All tests were performed following the manufacturer's instructions. The cut-off values for IgG and IgM seropositivity were obtained by firstly multiplying the mean optical densities of IgG and IgM calibrators by the correction factor (0.50) of the calibrator to obtain the corrected mean cut-off value; secondly, the CMV G and M indexes were calculated by dividing the optical density of each sample by the corrected mean cut-off value. A serum sample was considered positive for IgG or IgM antibodies when a CMV G index or a CMV M index was greater than 1.1, respectively. Negative and positive controls were included in each run. Samples positive for IgM by EIA were further tested by a commercially available enzyme linked fluorescent assay (ELFA): "CMV IgM Vidas" (BioMériux, France).

Statistical analysis

We performed the statistical analysis with the aid of the software Epi Info version 7, and SPSS 15.0 (SPSS Inc., Chicago, Illinois). For calculation of the sample size, we used a 95% confidence level, a power of 80%, a 1:1 proportion of cases and controls, a reference seroprevalence of 65.6% [27] as the expected frequency of exposure in controls, and an odds ratio of 2.3. The result of the sample size

calculation was 134 cases and 134 controls. We compared the age values among the groups by the paired Student's *t* test. The Pearson's χ^2 test and the two-tailed Fisher's exact test (when values were small) were used to assess the association between CMV seropositivity and clinical data of the pregnant women. We calculated the odds ratio (OR) and 95% confidence interval (CI), and a *P* value of <0.05 was considered statistically significant.

Ethics statement

We used only archival serum samples and clinical data from a previous study [26]. The original study was approved by the Institutional Ethical Committee of the General Hospital of the Secretary of Health in Durango City, Mexico. A written informed consent was obtained from all participants.

Results

Anti-CMV IgG antibodies were found in 138 (94.5%) of the 146 controls and in 136 (93.2%) of the 146 cases (OR = 0.78; 95% CI: 0.30–2.05; *P* = 0.62). High (>18 IU/ml) levels of anti-CMV IgG antibodies were found in 52 (37.7%) of the 138 seropositive controls and in 47 (34.6%) of the 136 seropositive cases (OR = 0.87; 95% CI: 0.53–1.43; *P* = 0.59). Anti-CMV IgM antibodies were found in 22 (15.1%) of the 146 controls and in 15 (10.3%) of the 146 cases by using EIA. These EIA IgM positive samples were further tested by ELFA. Anti-CMV IgM antibodies were found in 1 (0.7%) of the 146 controls but in none of the 146 cases using ELFA (*P* = 1.0). Seroprevalence of CMV infection was similar among cases regardless the diagnosis: mild preeclampsia (23/27: 85.2%), severe preeclampsia (89/95: 93.7%), eclampsia (16/16: 100%), and HELLP syndrome (8/8: 100%) (*P* = 0.21).

Seropositivity to CMV did not vary with age (*P* = 0.58). In addition, seropositivity to CMV was not associated (*P* > 0.05) with number of pregnancies, history of deliveries, cesarean sections, miscarriages, stillbirths, or a previous preeclampsia (*Table 1*).

Systolic blood pressure (157.98 ± 17.93 mmHg) in CMV positive cases was similar to that (157.00 ± 21.10 mmHg) in CMV negative cases (*P* = 0.86). Diastolic blood pressure (103.42 ± 10.44 mmHg) in CMV positive cases was similar to that (104.00 ± 12.64 mmHg) in CMV negative cases (*P* = 0.86). There was no difference (*P* = 0.96) in the mean arterial blood pressure among seropositive and seronegative cases (121.64 ± 11.77 mmHg and 121.80 ± 14.82 mmHg, respectively).

Discussion

Infection with CMV has been associated with essential hypertension [28, 29]. In a meta-analysis of three studies, researchers found a significant association between CMV and essential hypertension [29]. However, studies on the association between CMV infection and hypertensive disorders in pregnancy have shown conflicting results [20–25]. Therefore, the present study aimed to determine whether CMV infection is associated with hypertensive disorders in a sample of pregnant women in Durango City, Mexico. Our results of tests including qualitative detection of anti-CMV IgG antibodies, quantitative measure of anti-IgG antibody levels, and qualitative detection of

Table 1. Bivariate analysis of clinical characteristics and rates of IgG seropositivity to CMV in cases

Characteristics	Women tested	Seroprevalence of CMV infection		*P* value
	No.	No.	%	
Pregnancies				
1	75	70	93.3	0.25
2	31	28	90.3	
3	18	17	94.4	
4	8	8	100.0	
5	11	11	100.0	
6	1	1	100.0	
7	2	1	50.0	
Trimester of pregnancy				
2	2	2	100.0	1.00
3	144	134	93.1	
Deliveries				
Yes	65	60	92.3	0.75
No	81	76	93.8	

Table 1. *(cont'd)*

Characteristics	Women tested	Seroprevalence of CMV infection		*P* value
	No.	No.	%	
Cesarean section				
Yes	102	97	95.1	0.16
No	44	39	88.6	
Miscarriages				
Yes	25	25	100.0	0.21
No	121	111	91.7	
Stillbirths				
Yes	3	2	66.7	0.19
No	143	134	93.7	
History of preeclampsia				
Yes	21	19	90.5	0.63
No	125	117	93.6	

anti-CMV IgM antibodies indicate that seropositivity to CMV was equally observed in patients suffering from hypertensive disorders of pregnancy and age-matched pregnant women without these disorders attended in the same hospital. Stratification by clinical types of hypertensive disorders did not show a link between CMV and mild preeclampsia, severe preeclampsia, eclampsia, and HELLP syndrome. Thus, our results do not support an association between CMV infection and hypertensive disorders of pregnancy. Our results conflict with those reported in three previous studies. In a Canadian study, researchers found a significant increase of CMV seropositivity and higher anti-CMV IgG antibody levels in women with preeclampsia than normal pregnancy controls [24]. In another study, Xie et al. found that women with early-onset preeclampsia with HELLP syndrome had a significantly higher CMV IgG seropositivity rate than matched normal and non-pregnancy controls [25]. In addition, women with early-onset preeclampsia had higher anti-CMV IgG antibodies than women with late-onset preeclampsia and normal pregnancy [23]. In contrast, our results agree with those reported by other researchers. Soydinc et al. found that anti-CMV IgG and IgM antibodies seropositivities were not significantly different between pregnant women with preeclampsia and healthy pregnant women [20]. In a Chinese study of 52 pregnant women with preeclampsia and 34 women with uncomplicated pregnancy in their third trimester, seroprevalence of recent and long-dated CMV infections was similar in women with preeclampsia and women with normal pregnancy [21]. In a Norwegian study, no evidence of an effect of CMV IgG seropositivity on the likelihood of developing preeclampsia was found [22].

We found a considerable number of serum samples with positive results of anti-CMV IgM antibodies using EIA. False positive results have been reported in anti-CMV IgM antibody tests [30]. Therefore, to increase the specificity of IgM seropositivity, we used two methods to test for anti-CMV IgM antibodies (EIA and ELFA). Results of ELFA yield only one positive sample for IgM antibodies. The sample was from a control women, and thus, no association of this infection marker with hypertensive disorder of pregnancy was found. This result is in line with the lack of association of CMV IgM seropositivity and preeclampsia reported in a Turkish study [20] and a Chinese study [21].

Our study has some limitations. We enrolled a relatively small cohort of pregnant women, and subgroups of hypertensive disorders were also small. Women from only one public hospital were included in the study. Most women attended in the participating hospital have a low socioeconomic status. Therefore, further studies with a larger sample size, including women from several hospitals, with a higher number of participants with several types of hypertensive disorder of pregnancy and with a variety of socioeconomic status, should be conducted.

Conclusions

No serological evidence of an association between CMV infection and hypertensive disorders of pregnancy in patients in a public hospital in Durango City, Mexico was found. Our results conflict with those reported in previous studies. Therefore, further research to elucidate the role of CMV in hypertensive disorders in pregnancy should be conducted.

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Competing interests

The authors declare that no competing interests exist.

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BMC Infectious Diseases

RESEARCH ARTICLE Open Access

Lack of association between *Toxoplasma gondii* exposure and depression in pregnant women: a case-control study

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Abstract

Background: Very little is known about the link of *T. gondii* infection and depression. Through an age-, gender-, and month of pregnancy-matched case-control study, we determined the association of *T. gondii* infection and depression in pregnant women.

Methods: We studied 200 pregnant women with depression and 200 pregnant women without depression attended in a public hospital in Durango City, Mexico. Pregnant women were tested for the presence of anti-*Toxoplasma* IgG antibodies using an enzyme-linked immunoassay (EIA), and IgG seropositive women were further tested for the presence of IgM using an EIA. IgM positivity by EIA was further analyzed by enzyme-linked fluorescence assay (ELFA).

Results: Anti-*T. gondii* IgG antibodies were found in 9 (4.5%) of the 200 cases and in 12 (6.0%) of the 200 controls (OR = 0.73; 95% CI: 0.30–1.79; *P* = 0.50). The frequency of high (>150 IU/ml) anti-*T. gondii* IgG levels was similar in cases and in controls (OR = 1.20; 95% CI: 0.36–4.01; *P* = 0.75). Two women were positive for IgM by EIA but both were negative by ELFA.

Conclusions: We did not find serological evidence of an association between *T. gondii* infection and depression in pregnant women attended in a public hospital in Durango City, Mexico. Since an association of *T. gondii* and depression in pregnancy has been reported in the U.S. previously, further research to elucidate the role of *T. gondii* in prenatal depression should be conducted.

Keywords: *Toxoplasma gondii*, Seroprevalence, Depression, Pregnant women, Case-control study

Background

Toxoplasma gondii (*T. gondii*) is a widely-distributed parasite [1], transmitted to humans by ingestion of raw or undercooked meat containing tissue cysts, and ingestion of food or water contaminated with oocysts shed by cats [2, 3]. Primary infections in pregnant women may result in vertical transmission leading to congenital infections and disease [4, 5]. Although most infections with *T. gondii* are asymptomatic, some infected individuals develop a disease called toxoplasmosis with involvement of eyes, lymph nodes and central nervous system [6, 7]. Immunocompromised individuals infected with *T. gondii* are at risk for a reactivation of the infection leading to a severe disease mainly of the central nervous system [8]. Infection with *T. gondii* has been linked to psychiatric disorders including schizophrenia [9, 10], bipolar disorder, obsessive-compulsive disorder, and addiction [9]. However, the link between *T. gondii* infection and depression is controversial. In a Cuban study of psychiatric patients, those suffering from depressive disorders had the highest frequency of reactivity to the toxoplasmin intradermal test [11]. However, in a population-representative birth-cohort of individuals in Dunedin, New Zealand, *T. gondii* seropositivity

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was not significantly associated with major depression [12]. Similarly, in a meta-analysis of 50 studies into *T. gondii* infection for major psychiatric disorders versus healthy controls, no association between *T. gondii* IgG seroprevalence and major depression was found [9]. The association of *T. gondii* infection and depression in pregnant women has been poorly studied. Groër et al. found that higher anti-*T. gondii* IgG titers in infected women in the USA were related to depression and anxiety during pregnancy [13]. We aimed to determine whether *T. gondii* infection is associated with depression in pregnant women attended in a public hospital in Durango City, Mexico.

Methods

Study design and population studied

We performed an age-, gender-, and month of pregnancy-matched case-control study of 200 pregnant women suffering from depression and 200 pregnant women without depression attended in a public hospital in Durango City, Mexico. This study was performed from March 2015 to February 2016. Inclusion criteria for enrollment of participants were: 1) pregnant women suffering from depression attending prenatal care consultations in the General Hospital of the Secretary of Health in Durango City; 2) aged 13 years and older; and 3) who accepted to participate in the study. Socioeconomic status was not a restrictive criterion for enrollment. Mean age in cases was 23.40 ± 8.36 (range 13–43) years old. Depressed pregnant women had 2–8 months of pregnancy (mean 6.5 ± 1.5 months). As a strategy to screen depression in pregnant women, validated Mexican versions of the Edinburgh postnatal depression scales (EPDS) (Additional file 1) were used in adults [14] and teenagers [15]. Pregnant women who screened positive for depression in the EPDS were further examined by a psychiatrist to confirm depression using the Diagnostic and Statistical Manual of Mental Disorders, Fifth edition criteria [http://www.dsm5.org/Pages/Default.aspx]. Control pregnant women were matched with cases for age. Controls were randomly selected, and they scored negative for depression in the EPDS. Inclusion criteria for enrollment of control pregnant women were: 1) pregnant women without depression attending prenatal consultations in the General Hospital of the Secretary of Health in Durango City; 2) aged 13 years and older; and 3) who accepted to participate in the study. Mean age in control women was 23.01 ± 7.55 (range 13–45) years old. Pregnant women without depression had 2–9 months of pregnancy (mean 6.7 ± 1.5 months). No statistically significant differences in age ($P = 0.62$), and month of pregnancy between cases and controls were found.

Detection of anti-*T. gondii* antibodies

Serum samples from participants were obtained and stored at −20 °C until analyzed. The presence of anti-*T. gondii* IgG antibodies was tested in sera using the commercially available enzyme immunoassay (EIA) kit "*Toxoplasma* IgG" (Diagnostic Automation/Cortez Diagnostics Inc., Woodland Hills, CA, USA). Anti-*T. gondii* IgG antibody levels were expressed as International Units (IU)/ml, and a cut-off for seropositivity of 8 IU/ml was used. Sera positive for anti-*T. gondii* IgG antibodies were further tested for anti-*T. gondii* IgM antibodies by using the commercially available EIA "*Toxoplasma* IgM" kit (Diagnostic Automation/Cortez Diagnostics Inc.). In addition, sera positive for anti- *T. gondii* IgM antibodies by EIA were further analyzed for these anti-*T. gondii* IgM antibodies using the commercially available enzyme-linked fluorescent assay (ELFA) kit "VIDAS Toxo IgM" (BioMérieux, Marcy-l'Etoile, France). All IgG and IgM assays were performed following the instructions of the manufacturers.

Statistical analysis

Analysis was conducted using the software Epi Info 7 and SPSS 15.0 (SPSS Inc. Chicago, Illinois). For calculation of the sample size, we used a 95% confidence level, a power of 80%, a 1:1 proportion of cases and controls, a reference seroprevalence of 6.1% [16] as the expected frequency of exposure in controls, and an odds ratio of 2.8. The result of the sample size calculation was 195 cases and 195 controls. We used the student's *t* test to compare the age among cases and controls. The association of *T. gondii* infection and depression in pregnant women was assessed with the two-tailed Pearson's chi-squared test. Odds ratio (OR) and 95% confidence interval (CI) were calculated, and a P value < 0.05 was considered statistically significant.

Results

Of the 200 cases of depression included in the study, 122 (61.0%) suffered from minor depression, and 78 (39.0%) from major depression. Anti-*T. gondii* IgG antibodies were found in 9 (4.5%) of the 200 cases and in 12 (6.0%) of the 200 controls. The seroprevalence of *T. gondii* infection was similar in cases and in controls (OR = 0.73; 95% CI: 0.30–1.79; $P = 0.50$). Of the 9 anti-*T. gondii* IgG positive cases, 6 (66.7%) had IgG levels higher than 150 IU/ml, one (11.1%) between 100 and 150 IU/ml, and 2 (22.2%) between 8 and 99 IU/ml. In contrast, of the 12 anti-*T. gondii* IgG positive controls, 5 (41.7%) had IgG levels higher than 150 IU/ml, one (8.3%) between 100 and 150 IU/ml, and 6 (50.0%) between 8 and 99 IU/ml. The frequency of high (>150 IU/ml) anti-*T. gondii* IgG levels was similar in cases and in controls (OR = 1.20; 95% CI: 0.36–4.01; $P = 0.75$). Seroprevalence of *T. gondii* infection in patients with minor depression (4/122: 3.3%) was comparable to that (5/78: 6.4%) found in patients with major depression ($P = 0.29$). Stratification by age

groups (13–30 years, and older than 30 years) did not show differences ($P > 0.05$) in seroprevalences among cases and controls (3/149: 2.0% versus 10/159: 6.3%, and 6/51: 11.8% versus 2/41: 4.9%, respectively). Stratification by month of pregnancy groups (2–5 months, and more than 5 months) did not show differences ($P > 0.05$) in seroprevalences (2% versus 8.7%, and 5.4% versus 5.2%, respectively) or high IgG antibody levels (2% versus 4.3%, and 3.4% versus 1.9%, respectively) among cases and controls. None of the 9 anti-*T. gondii* IgG seropositive cases was reactive to anti-*T. gondii* IgM antibodies by EIA. Whereas 2 of the 12 anti-*T. gondii* IgG seropositive controls were reactive to IgM by EIA. These 2 IgM positive sera by EIA were negative to IgM by ELFA. Thus, none of the cases and controls was considered seropositive to IgM.

Discussion

Studies about the association of *T. gondii* infection and depression have shown conflicting results [9, 11, 12]. In addition, the association of *T. gondii* infection and prenatal depression has been poorly studied in particular. Therefore, the present study aimed to determine whether *T. gondii* infection is associated with depression in a sample of pregnant women in Durango City, Mexico. Results of tests for detection of *T. gondii* performed in the present study included qualitative and quantitative IgG and IgM assays. Our results do not point towards an increased rate of depression in pregnant women infected with *T. gondii* compared to matched control patients attended in the same hospital. We are aware of only one study on the link of *T. gondii* infection and depression in pregnancy. In such study, 414 women at 16–25 weeks of gestation in the USA were examined, and researchers found that higher *T. gondii* IgG antibody titers were associated with prenatal depression [13]. Authors hypothesized that immune escape of *T. gondii* may occur due to immune changes in pregnancy, and this could cause depression through activation of indoleamine 2, 3-dehydroxylase resulting in serotonin decrease [13]. It is not clear why the association of infection and depression was found in pregnant women in the USA but not in pregnant women in the current study. Comparison of both studies was based on IgG seropositivity; however, IgM seropositivity was not compared because this marker for acute or recent infection was not determined in the American study. It is possible that differences in the characteristics of pregnant women among the studies may explain the differences in the association. In the U.S. study, researchers examined women at 16–25 weeks of pregnancy whereas we examined women at 2–9 months of pregnancy. Stratification by month of pregnancy groups (2–5 months, and >5 months) did not show an association of infection and prenatal depression in the current study. While our study did not find any association of infection with *T. gondii* and depression in pregnant women several studies have demonstrated a link between *T. gondii* infection and depression. *T. gondii* seropositivity correlated with the Center for Epidemiologic Studies Depression score, Profile for Mood States-depression, and total mood disturbance score in a study of women veterans in the USA [17]. The age of women in the latter study was higher than in our study. Experimental evidence exists that reactivation of chronic *T. gondii* infection in mice by an immunosuppressive regimen caused depression-like behaviors, specifically, reduced sucrose preference, and increased immobility in the forced-swim test [18]. Researchers of the latter study also observed an enhanced tryptophan catabolic shunt and serotonin turnover that may be involved in the development of the depressive-like behaviors [18]. Reactivation of latent infection in humans is often observed in immunocompromised patients leading to life-threatening toxoplasmic encephalitis; it is therefore difficult to study an association with depression.

False positive results have been reported in anti-*T. gondii* IgM antibody tests [19]. Therefore, to increase the specificity of IgM seropositivity, we used two methods to test for anti-*T. gondii* IgM antibodies (EIA and ELFA). No acute cases of *T. gondii* infection were found, and therefore, treatment against *T. gondii* in the pregnant women studied was not needed.

Our study has limitations. First, we investigated the association of infection with *T. gondii* and depression in a relatively small cohort of pregnant women attending a public hospital. Therefore, our results cannot be extrapolated to pregnant women with different social status, i.e., those attended in private hospitals or other public hospitals. The great majority of women attended in the participating hospital had a low socioeconomic status.

Conclusions

We did not observe serological evidence of an association between *T. gondii* infection and depression in pregnant women attended in a public hospital in Durango City, Mexico. Our results conflict with those reported in a previous study in the USA therefore warranting further research to elucidate the role of *T. gondii* in prenatal depression.

Additional file

Additional file 1: Tool used to screen depression. (DOCX 15 kb)

Abbreviations

CI: Confidence interval; EIA: Enzyme-linked immunoassay; ELFA: Enzyme-linked fluorescence assay; EPDS: Edinburg postnatal depression scale; IU: International units; ml: Milliliter; OR: Odds ratio; SPSS: Statistical package for the Social Sciences; USA: United States of America

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Authors' contributions
CAE, LFSA, and JHT designed the study protocol, performed the data analysis and wrote the manuscript. ALMM, and JMCO obtained blood samples, submitted the questionnaires, and performed the data analysis. CSM and ASA performed the clinical assessment of participants. OL performed the data analysis, and wrote the manuscript. CAE, AASC, JMSP, and EIAS performed the laboratory tests. All authors read and approved the final version of the manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The Ethics Committee of the General Hospital of the Secretary of Health in Durango City, Mexico approved this study, and written informed consents were obtained from all participants and from the next of kin of minor participants.

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Lack of Association Between *Toxoplasma gondii* Infection and Diabetes Mellitus: A Matched Case-Control Study in a Mexican Population

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Abstract

Background: Very little is known about the association between infection with Toxoplasma gondii (*T. gondii*) and diabetes mellitus. We perform an age- and gender-matched case-control study to determine the association of *T. gondii* infection and diabetes mellitus.

Methods: Cases included 156 patients with diabetes mellitus and 156 controls without diabetes mellitus who attended in two public clinics in Durango City, Mexico. Sera of cases and controls were tested for the presence of anti-*Toxoplasma* IgG and IgM antibodies using commercially available enzyme-linked fluorescence assays (ELFA).

Results: Anti-*T. gondii* IgG antibodies were found in 10 (6.4%) of the 156 cases and in five (3.2%) of the 156 controls (odds ratio (OR): 2.06; 95% confidence interval (CI): 0.69 - 6.19; P = 0.18). The frequency of high (> 150 IU/mL) anti-*T. gondii* IgG levels in seropositive cases (1/10: 10.0%) was comparable to the one (1/5: 20%) in seropositive controls (OR: 0.44; 95% CI: 0.02 - 9.03; P = 1.00). None of the 10 cases and five controls with seropositivity to anti-*T. gondii* IgG antibodies were positive for anti-*T. gondii* IgM antibodies. Stratification by gender showed similar frequencies of *T. gondii* infection in female cases (7/107: 6.5%) and female controls (4/107: 3.7%) (OR: 1.80; 95% CI: 0.51 - 6.34; P = 0.53), and in male cases (3/49: 6.1%) and male controls (1/49: 2.0%) (OR: 3.13; 95% CI: 0.31 - 31.19; P = 0.61).

Conclusions: We conclude that there is not serological evidence of an association between *T. gondii* infection and diabetes mellitus in the studied subjects in Durango City, Mexico. Further studies to elucidate the role of *T. gondii* in diabetes should be conducted.

Keywords: *Toxoplasma gondii*; Seroprevalence; Diabetes mellitus; Case-control study

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Introduction

Toxoplasma gondii (*T. gondii*) is a coccidian parasite causing infections all around the world [1]. There are two main routes of *T. gondii* transmission to humans: ingestion of food or water contaminated with oocysts shed by *T. gondii* infected cats, and eating raw or undercooked meat containing tissue cysts [2, 3]. Vertical transmission may also occur when a primary infection is acquired during pregnancy [2, 4]. Most *T. gondii* infections are asymptomatic [5]. Some infected individuals may develop clinical manifestations of toxoplasmosis including lymphadenopathy, chorioretinitis, and meningoencephalitis [2, 5, 6]. A reactivation of a *T. gondii* infection in immunocompromised patients may lead to a life-threatening disease with involvement of the central nervous system [2, 5]. After infection, *T. gondii* spreads to many organs of the host [7]. The presence of *T. gondii* infection in pancreas has been reported in humans and animals. In humans, *T. gondii* infection may cause pancreatitis [8]. In a series of 18 autopsy cases of acquired toxoplasmosis in New York City, three cases had dissemination of *T. gondii* to pancreas [9]. In fatal toxoplasmic pancreatitis in AIDS patients, autopsies demonstrated pancreatic necrosis with free parasitic forms or tachyzoites [10], and cysts [11]. In animals, fatal acute toxoplasmosis involving pancreas has been observed in experimentally infected mice [12], a naturally infected Valley quail [13], a sand fox [14], and 11 sugar gliders [15].

It is unclear whether involvement of pancreas during *T. gondii* infection may lead to diabetes mellitus. In a Korean study, diabetes mellitus was a major coincidental disease in

T. gondii IgG seropositive patients [16]. In contrast, significantly lower levels of antibodies against *T. gondii* were found in Colombian patients with type 1 diabetes mellitus [17]. In a prospective cohort of Latino elderly in the USA, individuals seropositive to *T. gondii* did not show an increased rate of diabetes [18]. In an age- and gender-matched case-control study of 91 diabetic cases and 93 healthy non-diabetic controls in Iran, researchers found a higher seroprevalence of IgG antibodies against *T. gondii* in diabetic patients than in healthy controls [19]. In a meta-analysis of studies on the association between chronic toxoplasmosis and diabetes mellitus, researchers found that chronic toxoplasmosis was a possible risk factor for type 2 diabetes mellitus, and no association between *T. gondii* and type 1 diabetes mellitus [20]. Since results of studies about the association of *T. gondii* infection and diabetes mellitus are controversial, we decided to perform a matched case-control study to determine whether *T. gondii* seropositivity is associated with diabetes mellitus in subjects attending in a public health institution in Durango City, Mexico.

Materials and Methods

Study design and populations studied

We performed an age- and gender-matched case-control study of 156 patients with diabetes mellitus and 156 individuals without diabetes mellitus. Patients were enrolled in a public clinic for diabetes care (Clinica de Diabetes), and controls were enrolled in a public health center (Centro de Salud de Servicios Ampliados 450) in Durango City, Mexico. Both clinics belong to the same health institution (Secretaria de Salud). This study was performed from February 2015 to March 2017. Inclusion criteria for enrollment of cases were: 1) individuals with diabetes mellitus attending in a public diabetes care center (Clinica de Diabetes) in Durango City, Mexico; 2) aged 18 years and older; and 3) who accepted to participate in the study. Gender, occupation and socioeconomic status were not restrictive criteria for enrollment. In total, 107 (68.6%) females and 49 (31.4%) males with diabetes mellitus were enrolled in the study. Mean age in cases was 56.0 ± 11.1 (range 18 - 83) years old. Of the 156 patients, 151 had type II diabetes and five had type I diabetes. Control individuals were matched with cases for age and gender. Control individuals were randomly selected. Inclusion criteria for enrollment of controls were: 1) individuals without diabetes mellitus attending in public health center of the Secretary of Health (Centro de Salud de Servicios Ampliados 450); 2) aged 18 years and older; and 3) who accepted to participate in the study. The control group included 107 (68.6%) females and 49 (31.4%) males. Mean age in control subjects was 55.5 ± 11.8 (range 20 - 85) years old. Cases and controls had similar age (P = 0.70).

Detection of anti-*T. gondii* IgG and IgM antibodies

Serum samples from cases and controls were obtained and kept frozen at -20 °C until analyzed. Serum samples were analyzed for anti-*T. gondii* IgG antibodies using the commercially available enzyme-linked fluorescent assay (ELFA) kit "VIDAS Toxo IgG II" (BioMerieux, Marcy-l'Etoile, France). All serum samples with positive results in the IgG ELFA were further tested for anti-*T. gondii* IgM antibodies using the commercially available ELFA kit "VIDAS Toxo IgM" (BioMerieux, Marcy-l'Etoile, France). Both IgG and IgM ELFA were performed following the manufacturer's instructions.

Statistical analysis

Analysis of data was performed using the software Epi Info 7 and SPSS 15.0 (SPSS Inc., Chicago, IL). For the sample size calculation, we used the following values: a 95% confidence level, a power of 80%, a 1:1 proportion of cases and controls, and a reference seroprevalence of 6.1% [21] as the expected frequency of exposure in controls. Thus, a sample size of 155 cases and 155 controls was obtained. We used the Student's *t*-test to compare age values among cases and controls. The association between *T. gondii* infection and diabetes mellitus was analyzed with the two-tailed Pearson's Chi-squared test or the Fisher exact test (for small values). Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated, and statistical significance was set at a P value less than 0.05.

Ethics aspects

The Ethics Committee of the General Hospital of the Secretary of Health in Durango City, Mexico approved this study. Participation in the study was voluntary, and a written informed consent was obtained from all participants.

Results

Anti-*T. gondii* IgG antibodies were found in 10 (6.4%) of the 156 cases and in five (3.2%) of the 156 controls. The seroprevalence of *T. gondii* infection in cases was similar to the one in controls (OR: 2.06; 95% CI: 0.69 - 6.19; P = 0.18). Of the 10 anti-*T. gondii* IgG positive cases, one (10.0%) had IgG levels higher than 150 IU/mL, and nine (90.0%) between 8 and 99 IU/mL. Whereas, of the five anti-*T. gondii* IgG positive controls, one (20.0%) had IgG levels higher than 150 IU/mL, and four (80.0%) between 8 and 99 IU/mL. The frequency of high (> 150 IU/mL) anti-*T. gondii* IgG levels in cases was similar to the one in controls (OR: 0.44; 95% CI: 0.02 - 9.03; P = 1.00). None of the 10 cases and five controls with seropositivity to anti-*T. gondii* IgG antibodies were positive for anti-*T. gondii* IgM antibodies by ELFA. Stratification by gender showed similar frequencies of *T. gondii* infection in female cases (7/107: 6.5%) and female controls (4/107: 3.7%) (OR: 1.80; 95% CI: 0.51 - 6.34; P = 0.53). The frequency of *T. gondii* infection in male cases (3/49: 6.1%) was similar to the one in male controls (1/49: 2.0%) (OR: 3.13; 95% CI: 0.31 - 31.19; P = 0.61). Of the 151 patients with type II diabetes, nine (6.0%) were positive to IgG against *T. gondii*, whereas one (20%) of the five patients

with type I diabetes had IgG antibodies against *T. gondii* (P = 0.28).

Discussion

Whether *T. gondii* infection is associated with diabetes mellitus is still a matter of controversy. A limited number of studies about this association exist, and have reported conflicting results. Therefore, we sought to determine whether *T. gondii* infection is associated with diabetes mellitus in a sample of adult patients attending in a public clinic for diabetes care in Durango City, Mexico. For this purpose, we assessed the frequency of IgG and IgM antibodies against *T. gondii*, and the anti-*T. gondii* IgG antibody levels. Results of the current study indicate that patients with diabetes mellitus have equal frequencies of IgG and IgM antibodies against *T. gondii*, and anti-*T. gondii* IgG antibody levels as age- and gender-matched control subjects without diabetes. Therefore, our findings based on serological methods do not support an association between diabetes mellitus and *T. gondii* infection. Our results agree with the low (9%) seroprevalence of *T. gondii* infection reported in Mexican Americans from San Antonio Texas, USA that suffered from high rates of obesity and type 2 diabetes mellitus [22], and with low levels of antibodies against *T. gondii* found in Colombian patients with type 1 diabetes mellitus [17]. In addition, our results are in line with the lack of association between *T. gondii* infection and incident diabetes found in a prospective cohort of Latino elderly in New York, USA [18]. On the other hand, findings in favor of an association between *T. gondii* seropositivity and diabetes mellitus include a high seroprevalence of *T. gondii* infection found in patients with diabetes mellitus in general hospitals in Daejeon, Korea [16], and a significantly higher seroprevalence of *T. gondii* infection in patients with diabetes than in healthy controls found in an age- and gender-matched case-control study in Iran [19]. In addition, a meta-analysis of seven studies about the association of chronic toxoplasmosis and diabetes mellitus concluded that chronic toxoplasmosis was a possible risk factor for type 2 diabetes mellitus [20].

For a fair comparison of our results obtained under an age- and gender-matched case-control study design, we searched for similar studies in the medical literature. We were able to find only one age- and gender-matched case-control study about the association of *T. gondii* seropositivity and diabetes mellitus. In such study, researchers found a positive association between *T. gondii* infection and diabetes in Iran [19]. It is not clear why there were differences in the associations among the studies. It is likely that differences in the characteristics of the studied populations among the studies might explain the differences in the association. For instance, differences in age and gender among the studied populations might influence the seroprevalence of *T. gondii* infection. We could not compare the age and gender variables of our studied population with those of the Iranian study because no description of these variables was found in the Iranian study. A clear difference among the studies was the laboratory methods used. We used ELFA to determine antibodies against *T. gondii* whereas an enzyme-linked immunosorbent assay was used in the Iranian study [19]. Sensitivity and specificity of the IgG tests used in the studies are comparable. A 100% sensitivity and a 99% specificity of the enzyme-linked immunosorbent assay used were reported in the Iranian study [19]. According to the insert, the ELFA used in our study has a 99.65% sensitivity and a 99.92% specificity. The numbers of cases and controls were different among the studies. We enrolled 156 cases and 156 controls whereas 91 cases and 93 controls were included in the Iranian study [19].

The present study has limitations: we studied only diabetic outpatients from a single public clinic for diabetes care. The great majority of patients attending in this clinic have low socioeconomic status. The severity of diabetes in outpatients may be milder than in inpatients. Therefore, additional studies with diabetic inpatients, of diverse socioeconomic status, in several clinics should be conducted to further determine the association between *T. gondii* infection and diabetes mellitus.

Conclusions

We conclude that there was not serological evidence of an association between *T. gondii* infection and diabetes in adult patients attending in a public diabetes care center in Durango City, Mexico. Further studies to elucidate the role of *T. gondii* in diabetes should be conducted.

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Competing Interests

The authors declare that no competing interests exist.

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RESEARCH ARTICLE

Depletion of Hypocretin/Orexin Neurons Increases Cell Proliferation in the Adult Subventricular Zone

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Abstract: ***Background & Objective***: Adult neurogenesis, a specific form of brain plasticity in mammals that occurs in the subventricular zone, is subject to complex regulation. Hypocretin/orexin neurons are implicated in the regulation of sleep and arousal states, among other functions. Here we report for the first time the presence of orexinergic projections within the adult rat subventricular zone. Post-mortem retrograde tracing combined with immunofluorescence indicated orexinergic projections toward the subventricular zone. To establish the relationship between the depletion of orexin neurons and the number of proliferating cells in the subventricular zone, we labeled mitotic cells. Histological analysis revealed proliferating cells to be in close contact with orexinergic fibers. Neurotoxin-lesioning of orexin neurons in the lateral hypothalamus significantly activated precursor cell proliferation in the subventricular zone. Furthermore, cell proliferation in both normal and lesioned animals failed to reveal newly born orexin neurons in the lateral hypothalamus.

Conclusion: Based on these findings, we suggest that the adult subventricular zone is affected by orexinergic signaling, the functional implication of which must be further elucidated.

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1. INTRODUCTION

In 1998 two independent research groups discovered a class of hypothalamic peptides with neuroexcitatory activity [1, 2]. One group called these peptides "hypocretins" because they are produced ("secreted") in the hypothalamus [1]; the other group termed these peptides "orexins" (from the Greek: orexis = appetite) because they regulate feeding behavior. The neurons producing these peptides, however, are not exclusively related to feeding but also to sleep and waking behaviour, among other functions. As such, they are now termed "the hypocretin/orexin system". Orexin neurons are located exclusively in the lateral hypothalamus in rodents and humans, particularly in the perifornical, dorsomedial, and lateral portions [3, 4]. Orexin fibers widely project throughout the brain and have excitatory effects on their postsynaptic targets [5].

The sleep disorder narcolepsy is now widely considered a neurodegenerative disease, and is associated with a massive reduction in the number of neurons containing orexin [6-8]. Patients with narcolepsy have a severe reduction in the levels of orexin in the cerebrospinal fluid [9], a finding consistent with the loss of orexin neurons [10-13]. Lesioning of orexin neurons in rats also results in a decline in orexin levels in the cerebrospinal fluid, with these animals displaying narcoleptic-like symptoms [6, 14, 15]. Consistent with this evidence, patients with traumatic brain injury in the hypothalamus exhibit excessive daytime sleepiness and a concomitant loss of orexin neurons [12]. Moreover, orexin knockout mice exhibit narcolepsy [16]. Further, optogenetics

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has been employed to demonstrate a causal relationship between increased orexin neuron activity and wakefulness. Optogenetic photostimulation of orexin neurons enhances the probability of transition to wakefulness from sleep [17]. The above-mentioned studies thus support the hypothesis that orexin neurons play a functional role in the regulation of sleep and arousal states. A reduction in the number of orexin neurons produces sleep or narcolepsy, while an increase in the activity of orexin neurons produces wakefulness and arousal states.

Orexin neurons are also involved in learning and memory [18], as well as in cognitive functions and social interaction. For instance, nasal administration of orexin-A alleviates cognitive deficits induced by sleep deprivation and restores social memory in transgenic mice [19]. Recently, it was reported that orexin-A ameliorates learning and memory deficits and enhances hippocampal neurogenesis in epileptic rats [20]. Although neurogenesis induced by orexin-A was found in the hippocampus of epileptic rats, to our knowledge there are no studies about the relationship between orexin neurons and the other neurogenic brain region, namely, the subventricular zone (SVZ) of the lateral ventricles.

New neurons are continually generated in the adult mammalian brain. Adult neural stem cells (NSCs) reside in two neurogenic brain regions --the SVZ of the lateral ventricles and the subgranular layer of the hippocampal dentate gyrus [21, 22]. *In vivo* studies have shown that various growth factors, neurotransmitters, injuries, and degenerative neurological diseases affect the proliferation, migration, and differentiation of adult NSCs [21-25]. Recent evidence suggests that NSCs also exist in the subependymal layer (SEL) of the adult third ventricle [24]. Furthermore, intracerebroventricular injection of basic fibroblast growth factor and growth factor I increased proliferation of these progenitor cells and generated orexin neurons in the hypothalamus [26, 27]. Here, we investigate orexinergic innervation into the SVZ and examine the relationship between orexin neuronal cell loss and cell proliferation in the adult SVZ in a model of narcolepsy.

2. MATERIALS AND METHODS

2.1. Animals

Twenty-eight male Wistar rats (300-400 g) were housed at constant temperature (21±1°C) under a controlled light-dark cycle (lights on 7 AM-7 PM). Food and water were provided ad libitum. The experimental protocols were approved by the Research and Ethics Committee of our hospital, in accord with domestic and international standards of animal welfare, including the Mexican Standards Related to Use and Management of Laboratory Animals (DOF. 129 NOM-062-Z00-1999) as well as the National Institutes of 130 Health (NIH Publication No. 80-23, revised 1996). Sixteen animals were used for hypothalamic-orexin lesion and twelve for retrograde post-mortem tracing.

2.2. Experiment 1: Orexinergic Innervation of the SVZ

2.2.1. Retrograde Post-mortem Tracing

Ten- to twelve-week-old male Wistar rats (n=12) and brains from transgenic mice expressing the green fluorescent protein (GFP) under the nestin promoter (n=6) were used [28]. The nestin-GFP brains served to independently confirm the closeness of orexinergic fibers to neurogenic cells in the adult SVZ, as this relationship has not been demonstrated previously. We performed tracing experiments only in rats because we concluded from pilot experiments that the mouse brain is too small to allow precise tracer injections in defined anatomical structures.

Rats received a single intraperitoneal injection (100 mg/kg body weight) of BrdU (Sigma, St. Louis, MO) dissolved in 0.9% NaCl to label cells in the S-phase of the cell cycle at the time of injection. After 2 h, the animals were sacrificed with ketamine (100 mg/kg) and xylazine (13 mg/kg) and perfused transcardially with ice-cold 0.1 mol/L phosphate-buffered saline (PBS) followed by 4% (wt/vol) paraformaldehyde (PFA) in 0.1 mol/L PBS. Brains were removed and immersed in 4% PFA for 24 h.

To identify the origin of orexinergic fibers innervating the SVZ, brains were micro-dissected at anterior, medial, and posterior levels of the SVZ using atlas-based coordinates (anterior SVZ: 10.7 mm, intermediate SVZ: 10.2 mm, posterior SVZ -0.3 mm, relative to bregma; Paxinos and Watson, 1998). Using a stereotaxic frame (Kopf Instruments, Tujunga, CA) and a stereo-microscope (SMZ-2T, Nikon, Nikkei, Japan), 50 nl of the fluorescent bidirectional tracer 1,1-dilinoleyl-3,3,30,30-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR) was injected at a rate of 5 nl/min with a NanoFil microinjection syringe attached to a Micro-4 injection pump controller (both World Precision Instruments) into the dorsal SVZ. Each rat received only a single unilateral injection. Histological inspection typically revealed a very small hole in the tissue at the site of needle injection.

2.3. Experiment 2: Cell Proliferation in the SVZ in a Model of Narcolepsy

2.3.1. Hypothalamic-orexin Lesion

The experiment consisted of two randomly assigned groups: the experimental group was injected with the neurotoxin Orexin-SAP (n=8), whereas the control group was injected with saline (n=8). As previously described [14, 15], 90 ng of Orexin-SAP conjugate (Advanced Targeting Systems, San Diego, CA) or pyrogen-free saline was stereotaxically injected into the lateral hypothalamus (3.2 caudal, 1.7 lateral to bregma, 8.1 ventral to the skull surface) of male Wistar rats under ketamine-xylazine anesthesia (87 and 13 mg/kg, respectively). Sixty days after the injection of Orexin-SAP, the animals were deeply anesthetized with pentobarbital (100 mg/kg i.p.) and perfused transcardially with 0.9% saline followed by phosphate-buffered 4% paraformaldehyde (pH 7.0). The brains were postfixed overnight, equilibrated in 30% sucrose, and stored at 4°C. Five series of coronal sections were cut at 30 μm on powdered dry ice (Cryostat, Leica Biosystems). Each set of coronal brain sections was incubated overnight at room temperature with primary antibody [rabbit anti-orexin-A (1: 60 000, Peninsula Laboratories, Inc., San Carlos, CA)]. After washing, sections were incubated with secondary antibody for 1 h (Chemicon; 1:500 dilution) and then reacted with avidin-biotin complex for 1 h (Vector Laboratories, Burlingame,

CA). The diaminobenzidine method was used to visualize the reaction product. The omission of the primary antiserum resulted in no specific staining.

2.4. Protocols for Studying adult Neurogenesis

To ensure the labeling of proliferating cells in the SVZ and lateral hypothalamic area, 5-bromo-2′-deoxyuridine (BrdU, 50 mg/kg, Sigma-Aldrich, St. Louis, Missouri) was injected intraperitoneally twice a day, from day 8 to day 14 following Orexin-SAP or saline injection. The animals were killed eight weeks after the injection of Orexin-SAP. After transcardial perfusion with 4% ice-cold phosphate-buffered PFA, brains were post-fixed in 4% PFA and sectioned in the coronal plane at 30 µm on powdered dry ice (Cryostat, Leica Biosystems). Free-floating sections were denatured by incubation in 2 N HCl (37°C, 20 min) before BrdU staining. The sections were then incubated for 48 h with a rat anti-BrdU antibody (mouse anti-BrdU Kit II; Roche Molecular Biochemicals, Indianapolis, IN; dilution 1:50) and rabbit anti-orexin-A (1: 60 000, Peninsula Laboratories, Inc., San Carlos, CA). After washing the sections were incubated for 2 h at room temperature with FITC-conjugated secondary anti-mouse antibody (provided with the anti-BrdU kit II) and a TRITC-conjugated anti-rabbit antibody (Zidmed, South San Francisco, CA; 1:250 dilution). Cross-reactivity of secondary antibodies was systematically checked by varying the order of staining in the different experiments. The sections were counterstained with 4′,6′-diamino-2-phenylindole-dihydrochloride (DAPI).

2.5. Analysis of Immunofluorescence Samples

All microscopic analyses were conducted in a blinded fashion. Orexin-labeled cells were counted with an epifluorescence microscope and an x40 objective. The observer counted the clearly stained orexin-immunoreactive (ir) somata in twenty sections (1 in 5 series) of both hemispheres, encompassing the full extent of orexin distribution (between -1.6 and -3.8 mm from bregma [13]. A double-labeled protocol with BrdU/Orexin was then counted and split into ten sections (2 in 5 series) of the left SVZ, between 0.4 to 1.0 mm from bregma. In the left SEL (subependymal layer) of the 3rd ventricle and left hypothalamus, a BrdU/Orexin double-labeled protocol was counted and split into ten sections (1 in 5 series) between -1.6 and -3.8 from bregma. Confocal fluorescence microscopy analysis was performed with an Olympus FV1000 (Tokyo, Japan) microscope, fitted with a 40x objective.

2.6. Statistical Analysis

Statistical analysis was performed using SigmaPlot (Systat Software Inc). All data are presented as means ± s.d. and were statistically evaluated using an analysis of Independent Group t-test followed by the Mann-Whitney U test. A value of $p<0.05$ was considered to be statistically significant.

3. RESULTS

3.1. Orexinergic Innervation within the Adult SVZ

Orexin-ir cells were found in the lateral hypothalamus/perifornical area, as described earlier [11] (Fig. **1a**). The number of orexin-ir cells in the control group was 1126.0 ± 79.6 per rat (n=8). A high density of orexin fibers was found in the septum (Fig. **1b**) and a very low density in the corpus callosum and striatum (Figs. **1b**). Interestingly, a high density of orexin afferents was localized around the lateral ventricle (Fig. **1b**; arrows), including the SVZ (Fig. **1c**, arrows), which is rich in adult NSCs [2]. Confocal microscopic analysis showed orexin fibers to be in close contact with BrdU-labeled SVZ cells in the saline group (Fig. **1d**, arrows).

To study the distribution of orexinergic afferents within the mouse SVZ, nestin-GFP mouse brain sections were used to visualize neural precursor cells. As shown in Fig. (**1i**), a relatively higher density of orexinergic fibers was found within the SVZ, where the nestin-GFP cells were located. Orexinergic fibers were particularly found close to somata of nestin-GFP cells (Fig. **1i**). These results corroborated that SVZ neurogenesis may be influenced by orexins.

3.2. Retrograde Tracing from the SVZ

A representative example of an injection particularly targeting the dorsal-SVZ is shown in Fig. (**2a**), identified anatomically by the presence of BrdU-labelled cells (blue) at the site of DiI injection (red). Scanning the lateral hypothalamic area (Fig. **2b**) demonstrated a cluster of orexin/DiI neurons in the lateral perifornical area. These results corroborated the orexinergic innervation of the SVZ from the medial-lateral hypothalamus.

3.3. Cell Proliferation in the SVZ in a Model of Narcolepsy

To study the effect of hypothalamic orexin lesion on the number of $BrdU^+$ SVZ cells, we selectively destroyed orexin innervation using the neurotoxin orexin-SAP. At 60 days after orexin-SAP injection, there was a significant decrease (53%) in the number of hypothalamic orexin-ir neurons (596.78 ± 225.4; n=8) in comparison to control group (Fig. **1e**). Loss of orexin-ir neurons was severe in the perifornical and lateral portions of the hypothalamus (Fig. **1e**). Measuring fiber density showed a 67% denervation *vs*. control in structures around the lateral ventricle (Fig. **1f**, arrows). Interestingly, the SVZ in the orexin-SAP group was consistently labelled with DAPI, with an increase in cell density compared to control group (Fig. **1g**). There was a decrease also in orexin fiber density in the SVZ (Fig. **1g**, arrows). BrdU-labeled cell number in the SVZ increased by 100.6% in the orexin-SAP group compared to the saline group (orexin-SAP, 661.6 ± 98.4; control, 329.7 ± 54.6; $p<0.001$) (Fig. **1h**,**j**).

We also investigated neurogenesis in the SEL of the 3rd ventricle and hypothalamus (Fig. **3**). Control rats displayed a high density of orexin fibers in the SEL, which were in close contact with the proliferating cells (Fig. **3a**). As expected, no BrdU/orexin double-labeled cells could be discerned in the hypothalamic parenchyma in controls (Fig. **3b**). Orexin fiber density in the SEL decreased 65% in orexin-SAP rats (Fig. **3c**). Cell proliferation in the latter group was significantly increased (about 196.6%) in terms of BrdU-labeled cell number in the SEL (Orexin-SAP, 436.7 ± 68.4; control, 147.2 ± 36.3; $p<0.001$) (Fig. **3e**). However, there was no evidence for new orexin neurons in either the hypothalamus or SEL (Fig. **3c** and **d**).

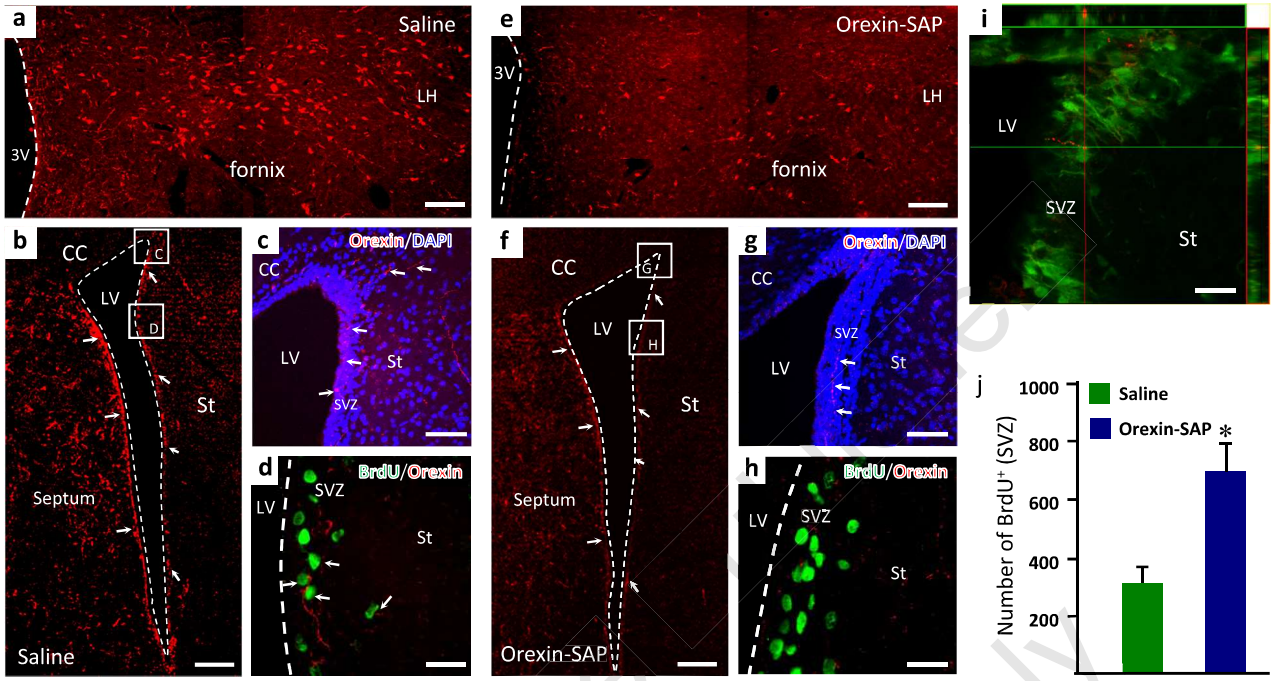

Fig. (1). Hypocretin/orexin depletion increases cell proliferation in the adult subventricular zone (SVZ). (**a**) Perifornical distribution of orexin neurons (red) in the adult lateral hypothalamus (LH). (**b**) Orexin afferents (red) in the septum, corpus callosum (CC) and striatum (St). High density can be seen around the lateral ventricle [arrows]. (**C**) Inset in b, orexin innervation in the SVZ (counterstained with DAPI in blue). (**d**) Inset in b, proliferating cells (BrdU-labelled, green) in close contact with orexin fibers (red). Animals were sacrificed 2 h following BrdU injection. (**E**) Severe loss of orexin neurons 8 weeks after injection of orexin-SAP. (**f**) Note significant orexin denervation around the lateral ventricle (arrows). (**g**) Inset in f, orexin denervation (arrows) increased cell density (DAPI staining) in the SVZ. (**h**) Orexin denervation was associated with precursor cell proliferation (BrdU-labelled; green) in the SVZ. (i) Confocal image of a nestin-GFP mouse, showing orexin-A fibers (red) in close vicinity to GFP precursor cells (green) in the adult SVZ. (**j**) A number of BrdU-labelled cells in the SVZ (*p< 0.001). Scale bar: a,e,c,g: 100 μm; b and f: 500 μm; d,h,j: 50 μm. (*The color version of the figure is available in the electronic copy of the article*).

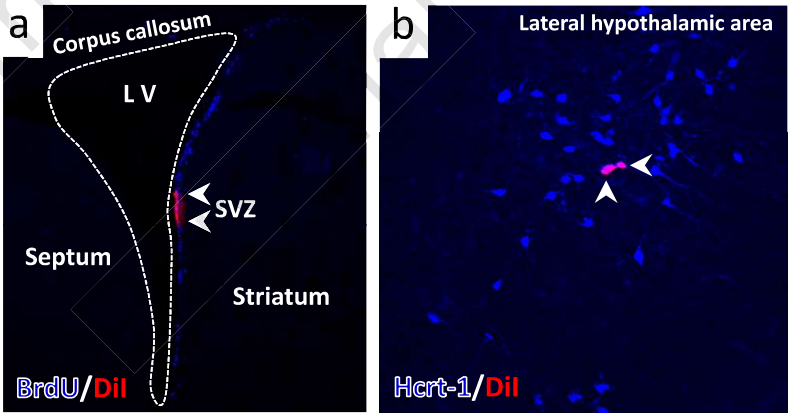

Fig. (2). Retrograde tracing from the dorsal subventricular zone (SVZ) to the lateral hypothalamic area. (**a**) Confocal photograph is showing the DiI (red) injection site in the SVZ, containing BrdU+ cells (blue), located between the lateral ventricle (LV) and the striatum (Str). The arrows indicate the anatomical location of the injection site at 0.2 mm anterior from bregma. (**b**) Confocal micrograph of the hypothalamic area showing orexinergic neurons (blue). Some of the orexin+ cells were DiI+. These data confirm connectivity of orexinergic neurons with the SVZ. (*The color version of the figure is available in the electronic copy of the article*).

4. DISCUSSION

In non-lesioned rats and mice, we found orexin neurons projecting to the SVZ of the lateral ventricles and the SEL of the third ventricle. However, in lesioned rats, the density of these projections was reduced. This latter finding was related to an increase in the number of proliferating precursor cells in both the SVZ of the lateral ventricles and the SEL of the third ventricle (Fig. **1j** and Fig. **3e**).

Here we show, for the first time, by histological means, that orexin neurons send axonal projections to the SVZ of the

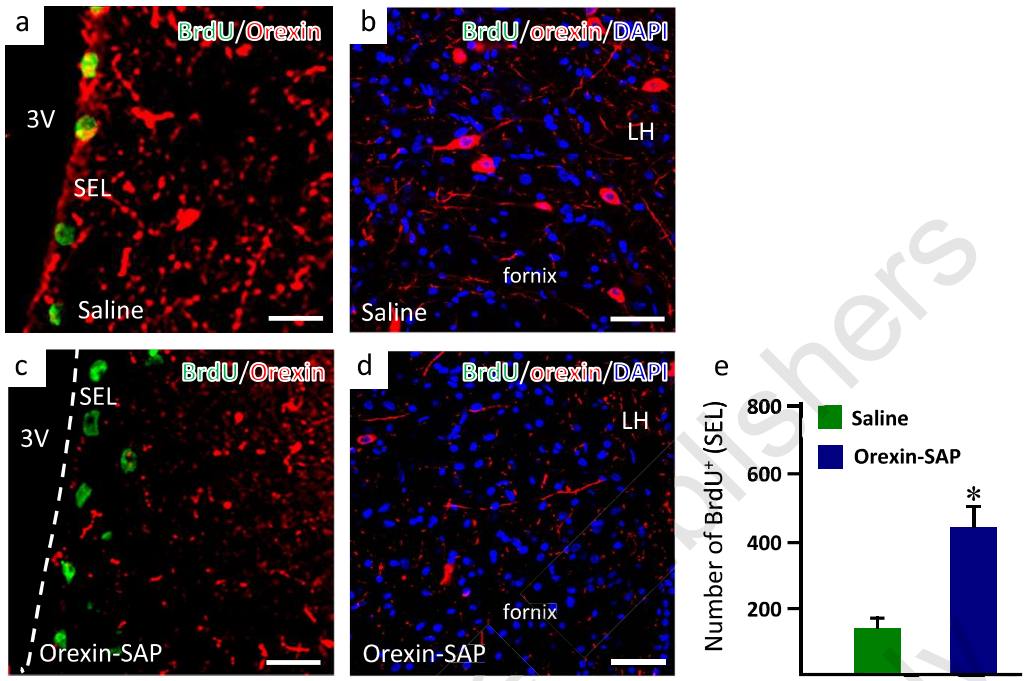

Fig. (3). Hypocretin/orexin depletion increases proliferation in the subependymal layer (SEL) of the third ventricle (3V). (**a**) Orexin fibers (red) in the SEL of 3V in close contact with BrdU-labelled cells (green). Note absence of double-labelled cells. (**b**) Orexin neurons in the lateral hypothalamus (LH) counterstained with DAPI; BrdU-labelled cells are absent. (**C**) Orexin denervation in the SEL of 3V. Numerous BrdU-labelled cells can be seen (green), with no evidence of double-labelled cells. (**d**) Orexin neuronal cell loss in the LH (red) counterstained with DAPI, with no evidence of BrdU-labelled cells. (**e**) A number of BrdU-labelled cells in the SEL (*p< 0.001). Scale bar: A and C: 50 µm. B and D: 100 µm. (*The color version of the figure is available in the electronic copy of the article*).

lateral ventricles. This result is consistent with pharmacological evidence that orexin neurons also project to the brain's other important neurogenic zone, the hippocampus [18-30]. Therefore, we suggest that hypothalamic orexin neurons send divergent projections to those neurogenic regions of the brain where adult NSCs reside.

Zhao *et al.* [20] showed that increased activation of orexin neurons by orexin-A in epileptic rats produced an increase in hippocampal neurogenesis. This apparent discrepancy with our finding that a reduction in orexin neurons in normal rats induced an increase in SVZ neurogenesis in the lateral ventricles could be explained by different animal models employed, and different neurogenic zones explored. Epileptic seizures can stimulate neurogenesis via regulatory steps that include proliferation, differentiation, and migration of newborn neurons in the subgranular zone of the dentate gyrus in the hippocampus. Therefore, it is possible that the increased neurogenesis observed by Zhao *et al.* in epileptic rats could be due, in part, to the epileptic seizures in the animal model employed [31-34]. One may thus speculate that orexin depletion in the hypothalamus could also increase neurogenesis in the hippocampus in normal rats, as in our study, in the SVZ of the lateral ventricles. Our findings are supported by studies showing that sleep deprivation in normal rats inhibits adult neurogenesis in the hippocampus and by studies that demonstrating that sleep facilitates adult hippocampal neurogenesis [21, 25].

The possible mechanism by which orexin neurons modulate neurogenesis in the SVZ of the lateral ventricles or hippocampus could involve regulation of cell proliferation and differentiation by activating extracellular kinase1/2 via orexin-1 and orexin-2 receptors, which activate multiple G-proteins [20]. *In vitro* studies on the effects of orexins in HEK-293 cells [35] and angiogenesis by neovascularization [36] support this possibility.

In the adult mammalian brain, the SVZ of the lateral ventricles is characterized by the presence of multipotential cells with persistent proliferation [5, 37]. An increase in proliferation and neurogenesis in the SVZ has been observed in neurological disorders such as cerebral ischemia [38], Huntington disease [39]; Alzheimer disease [10] and multiple sclerosis [40], while a reduction in proliferation found in Parkinson's disease was linked to dopamine depletion [41-44]. To the best of our knowledge, the present findings are the first to report an increase in precursor cell proliferation, in both the SVZ and SEL of the 3rd ventricle, following orexin depletion. No evidence for new orexin neurons was found in either normal or lesioned animals. These observations point to a heretofore unknown role of orexin, namely, adult neurogenesis.

A discussion on the possible functional roles of orexin neuronal projections to the SVZ should also include the targets of neurons born in the SVZ. One such target is the olfactory bulb (OB). New neurons born in the SVZ of adult animals migrate anteriorly to the OB, where they mature into local interneurons [21, 23]. In fact, there is an SVZ-OB system that forms a route for migrating cells in continuous movement throughout interconnected paths along the SVZ

and the rostral migratory stream [21, 23]. It is tempting to speculate that this process of migration through the SVZ-OB system could be facilitated when orexin neuronal projections induce an increase in SVZ precursor cell proliferation. On the other hand, this migration process could be interrupted when orexin neuronal projections decrease SVZ cell proliferation. This last possibility is consistent with two well-known facts. First, in Parkinson disease, there is a reduction in precursor cell proliferation in the SVZ. Second, in Parkinson disease, there is a dramatic olfactory loss and decreased olfactory bulb volume.

Based on our results we suggest that increased activity in the orexin-SVZ pathway could lead to a reduction in cell proliferation in the SVZ and a concomitant reduction of potential new cells in the OB. Thus, over-activation of the orexinergic system, as occurs in sleep deprivation, could lead to a reduction in adult SVZ neurogenesis and loss of cells in the OB. Indeed, we predict that decreased activity in the orexin-SVZ pathway (such as that produced by narcolepsy, or as demonstrated here by lesioning the lateral hypothalamus which is rich in orexin neurons), can lead to an increase of new OB cells with an associated increase in the volume of the OB. In this context, our findings are of importance because they allow us to predict new lines of research to understand the link between the orexinergic system, neurogenesis, the SVZ-OB system and associated brain pathologies.

LIST OF ABREVIATIONS

BrdU	=	5-Bromo-2´-deoxyuridine
NSCs	=	Neural stem Cells
OB	=	Olfactory Bulb
SEL	=	Subependymal Layer
SVZ	=	Subventricular Zone

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The experimental protocols were approved by the Research and Ethics Committee of our hospital, National Insitute of Health (DOF. 129 NOM-062-Z00-1999) as well as the National Institutes of 130 Health (NIH Publication No. 80-23, revised 1996).

HUMAN AND ANIMAL RIGHTS

No human were used in the study all reported animals were experimented in accordance with domestic and international standards of animal welfare, including the Mexican Standards Related to Use and Management of Laboratory Animals.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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AUTHOR'S CONTRIBUTIONS

Oscar Arias-Carrión conceived the experiments, performed the statistical analysis, and prepared the figures. All authors performed experiments, prepared data and wrote the manuscript.

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REVIEW ARTICLE

The Role of Innate Immune System Receptors in Epilepsy Research

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Abstract: ***Background & Objective***: Epilepsy is one of the most complex neurological disorders and its study requires a broad knowledge of neurology and neuroscience. It comprises a diverse group of neurological disorders that share the central feature of spontaneous recurrent seizures, and are often accompanied by cognitive deficits and mood disorder. This condition is one of the most common neurological disorders. Until recently, alterations of neuronal activities had been the focus of epilepsy research. This neurocentric emphasis did not address issues that arise in more complex models of epileptogenesis. An important factor in epilepsy that is not regulated directly by neurons is inflammation and the immune response of the brain. Recent evidence obtained in rodent epilepsy models supports the role of immune responses in the initiation and maintenance of epilepsy. Recognition of exogenous pathogens by the innate immune system is mediated by some pattern recognition receptors such as Toll-like receptors leading to cell activation and cytokine production. Currently, these receptors have been the focus of epilepsy studies looking to determine whether the innate immune activation is neuroprotective or neurotoxic for the brain.

Conclusion: Here, we present the evidence in the literature of the involvement of key innate immune receptors in the development of epilepsy. We address some of the contradictory findings in these studies and also mention possible avenues for research into epilepsy treatments that target these receptors.

Keywords: Cytokines, epilepsy, epileptogenesis, innate immune system, neurological, toll-like receptors.

1. INTRODUCTION

According to the World Health Organization [1], epilepsy is one of the most common and incapacitating neurological disorders. Epilepsy is defined by a predisposition toward the generation of spontaneous recurrent seizures (SRS), which have neurological, cognitive and psychological consequences [2]. There are over forty types of epilepsy, and the most common type, temporal lobe epilepsy (TLE), represents 60% of cases [3]. Although TLE is the most common epilepsy syndrome, its etiology is often a mystery as few patients experience an injury, such as head trauma or meningitis that the onset of epilepsy could be clearly attributed to. The partial seizures of TLE originate in the hippocampus, entorhinal cortex, and amygdala. These seizures may occur many years after an initial precipitating injury such as status epilepticus (SE), cerebral trauma, febrile seizures, hypoxia-ischemia, *etc*. [4-6]. The range of hypotheses proposed to explain spontaneous seizure generation, extend from the molecular to the network level.

Currently, the models used most often to study epileptogenic mechanisms in TLE are status epilepticus (SE) models. These involve inducing a prolonged seizure with electrical

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stimulation or chemoconvulsant administration (typically pilocarpine or kainic acid). This initial brain injury is followed, after a dormant period of days to weeks, by spontaneous recurrent seizures. SE models reproduce much of the pathology of human TLE [7]. The use of these models, as well as other tools provided by modern neuroscience and molecular biology, have changed many of the prevailing views in this field such as the neurocentric view of seizures and epilepsy. Research with these models continues to expand our knowledge of the mechanisms of this disorder.

An important factor in epilepsy that is not regulated directly by neurons is inflammation and the immune responses of the brain [8, 9]. Increasingly, evidence supports that immune responses play a critical role in the initiation and maturation of epilepsy [10-12]. A series of studies in animal models of epilepsy, later corroborated by tissue specimens from patients with epilepsy, showed that seizures cause a dramatic proinflammatory signal mediated by microglia secreting cytokines such as interleukins [13]. Chronic brain inflammation can potentially lower the seizure threshold, which may then promote neuronal excitability by modifying neuronal channels, altering neurotransmitter release or uptake, and producing changes in brain-blood barrier permeability [14-16].

Brain insult initiates a process in which the repetitive action of certain exogenous neuroactive substances lead to significant functional changes in the neural networks including dendritic spine changes, excitatory synaptogenesis, axonal sprouting, and aberrant neurogenesis. After an initial precipitating injury, proliferation, differentiation and ectopic migration of these cells increase. The endogenous neuroprotective mechanisms are activated and initiate change from growth factor release to inflammatory suppression or other neurotrophic roles. A malfunction in these homeostatic mechanisms has been proposed to lead to the development of chronic epilepsy.

Identification of exogenous pathogens by the innate immune system is mediated by some pattern recognition receptors such as Toll-like receptors (TLRs) leading to cell activation and cytokine production. In vertebrates, the family of TLRs sense a variety of bacterial and viral structures. TLR3, TLR7, and TLR9 belong to the subfamily of TLRs that are expressed in endosomal/lysosomal compartments and recognize nucleic acids such as double-stranded RNA, single-stranded RNA and CpG-DNA containing DNA, respectively. TLR3, TLR7, and TLR9 are expressed in microglial cells, but may also be found in invading immune cells [17]. Adding to the complexity is that neuronal progenitor cells (NPCs) also seem to express various TLRs. Also, most recently, TLR7 has been described a role in recognizing miRNA and thereby contributing to neuronal damage [18, 19]. Currently, different viral or microbial infection models are being studied to determine whether innate immune activation mediates inhibition of axonal regeneration, is neuroregenerative or neurotoxic for the brain.

The chain of events that lead to the establishment of chronic epilepsy is complex and multifactorial; angiogenesis, genomic and proteomic responses, and neurogenesis are all considered to be a part of the disorder. Here, we discuss the role of innate immune receptors such as TLRs in epilepsy research.

2. TEMPORAL LOBE EPILEPSY ANIMAL MODELS:

First, we will give an overview of the animal models currently used in this area of research.

As surgical specimens from epilepsy cases are collected at the chronic stage of the disease they are unlikely to reveal early features that are critical for the disease process; thus, animal models were developed as experimental paradigms to investigate the mechanisms underlying epilepsy. Different animal models have been developed to reproduce the changes that occur in humans and to replicate the histopathological, electroencephalographic and behavioral features of this neurological condition [20-22]. Once it had been proven that inducing SE in a rat produces a pattern of hippocampal neuronal loss similar to patients with temporal lobe epilepsy, a valuable animal model was established to understand underlying mechanisms of epileptogenesis and epilepsy. However, there are currently no rodent models that develop spontaneous seizures later in life. Nearly all animal models are based on "normal" rodents that receive a proconvulsant insult that produces brain injury and epilepsy; the lack of animal models that more closely mimic human epilepsy is part of the complexity inherent in the study of this disorder.

2.1. Pilocarpine and Kainate Models

PILO and KA are the typical "post-SE models of TLE" the administration of these chemoconvulsants in rodents instigates a pattern of repetitive limbic seizures and SE that may continue for several hours. Following SE, a seizure-free period follows (varying from days to weeks) in which epileptogenesis takes place. Later, the SRS can be observed, with a gradual and progressive increase in their frequency. This final stage is permanent and defines the chronic phase of epilepsy [6, 20, 21]. Although the neuropathological changes in PILO and KA models are similar to those reported in hippocampi from patients with hippocampal sclerosis, the damage in both these models tends to be more severe and widespread [4, 20, 21]. PILO and KA can also be used as acute seizure models when evaluated during SE or epileptogenesis, *i.e*., before the onset of SRS [23].

Kainate is a structural analog of glutamic acid and can be administered locally (intra-hippocampal "IHpKA") or systemically [3, 6, 24]. The KA model exhibits robust neuronal depolarization, cell death, and cell morphological changes, which are classic characteristics of TLE in humans [25]. Pilocarpine (PILO) is an agonist of muscarinic acetylcholine receptors. Its systemic or intracerebral injection produces structural damage and consequently, development of SRS that resembles those of human complex partial seizures [26]. There are also similarities between human TLE and the PILO model; for instance, neurotrophins are upregulated in the hippocampus, and cognitive, and memory deficits are commonly found [26, 27].

Once chronic epilepsy is established, it is possible to observe in an electroencephalogram, two distinct phases of abnormal neuronal firing in the brain, the interictal and the

ictal. In the interictal condition, large epileptiform spikes are seen focally, representing synchronized firing of several populations of hyperexcitable neurons. In the ictal state, where tonic-clonic seizures occur, a long run of spikes is observed due to the massive synchronization and spread of neuronal activity [28, 29]. Later, the seizure may stop, either spontaneously or after pharmacological intervention, this period is known as the post-ictal state. Finally, the interictal state returns, with its persistent, abnormal neuronal function [29].

2.2. Electrical and Chemical Kindling

Kindling has been used extensively as a chronic model of TLE; focal seizures are provoked with repetitions of sub-convulsive brain stimulation (most commonly electric) which produce clear progressive change that lowers the seizure threshold [30]. The kindling model initially produces focal seizures, however, with daily repetitions, seizures spread to involve other areas of normal neurons; this process is referred to as recruitment. Kindling can model complex partial seizures with secondary generalization. Ultimately, this model culminates in the emergence of SRS and establishment of full, clonic, motor seizures [21, 26, 30]. As the sequence of molecular and cellular alterations that are produced in neural circuits by kindling are quite strong and reproducible, this model is considered to be a very effective tool for the observation of seizure-induced plasticity and epileptogenesis mechanisms [26].

Chemical kindling is similar to electrical kindling, but in this case, the development of electrographic and behavioral seizures occurs gradually after repeated stimulation by pharmacological agents. These chemicals are the source of activation of the neural circuitry instead of the electrical stimulus [26]. Chemical kindling can be induced by administering low doses of excitatory agents such as pentylenetetrazol (PTZ), a γ-aminobutyric acid $(GABA)_A$ receptor antagonist directly into the brain.[31-33]. Also worthy of note, PTZ can be used to induce absence seizures if injections of low doses are repeated within a brief period (*i.e*., one hour) [26]. Additionally, if the maximal total dose of PTZ is delivered as a single bolus its administration can produce convulsions provides a first line of defense [26]. Therefore, the induction of convulsive or nonconvulsive behavior is dependant on the PTZ administration protocol.

3. INNATE AND ADAPTIVE IMMUNITY IN THE CENTRAL NERVOUS SYSTEM:

The function of the innate immune system to defend against exogenous pathogens is well known. However, its role in the central nervous system (CNS) is a relatively new field of research. The central nervous system once thought of as an immunologically privileged site, could now be more accurately defined as an immunologically specialized site, as evidence has shown that immune and inflammatory reactions do occur in the CNS. The immune system has evolved into two parts: the *innate immune system* (providing an early inflammatory response) which is responsible for immediate action against most infectious agents; however, it does not recognize all pathogens. The other, the *adaptive immune system* recognizes specific antigens and provides a more versatile defense *via* antigen presenting cells and receptors [10, 34].

The innate immune system is essential for controlling infection during the 4-7 day period before the adaptive immune response takes over. The phagocytic cells of the IIS, include microglia and monocytes/macrophages; whereas, the key cellular members of the adaptive immune system are B and T lymphocytes [34, 35]. Intercellular communication of the immune system occurs directly *via* cell-to-cell contact or by cytokines, soluble factors that regulate immune response and inflammation. After systemic infections, the brain activates an inflammatory response whose job it is to provide protection against infectious microorganisms. A large variety of inflammatory mediators such as cytokines and Toll-like receptors (TLRs) regulate the transition between innate and adaptive immunity [36].

3.1. Innate Immunity

Innate immunity is the immediate and nonspecific response to pathogens by the host. Activation of this first line of defense involves leukocytes including the granulocytes (neutrophils, eosinophils, and basophils) cells of the monomyelocytic lineage (monocytes, macrophages, and microglia), dendritic cells, and TLRs, which are transmembrane proteins expressed by immunocompetent cells such as antigen-presenting cells (APCs). TLRs and the interleukin (IL)-1 receptor family share common cytoplasmic domains and employ partly overlapping signaling molecules that have the IL-1 receptor type 1 [9, 15]. TLRs have a fundamental role in the recognition of conserved motifs common to pathogens as well as 'danger signals'; the endogenous molecules that are released from damaged or stressed cells. It is the activation of TLRs that trigger innate immune responses and inflammation in the case of infection or when tissue injury occurs [37, 38].

3.2. Adaptive Immunity

The adaptive immune system is activated in response to innate immunity. Its response includes the recognition and immunological memory of specific non-self antigens, which then initiate the production of antibodies or immune responses that are mediated by B and T lymphocytes. This processing of antigens by APCs is vital for an effective adaptive immune response. These cells display antigen complexed with major histocompatibility on their surfaces in a process known as antigen presentation. There are two categories of antigen-presenting cells: professional and non-professional. Professional APC express MHC class II molecules along with co-stimulatory molecules and pattern recognition receptors. The main types of professional APCs are macrophages, B cells, and dendritic cells. These and brain-resident microglia present foreign antigens to naive T cells. Antigen presentation relies on other specialized signaling molecules on the surfaces of both APCs and T-cells. A dysregulation of adaptive immunity has been implicated in a loss of tolerance to self-antigens that leads to the development of autoimmunity. Regulatory T cells ($CD4^+CD25^+$) are a subpopulation of T cells that are immunosuppressive. As they restrict autoimmune activity, they contribute to immune system homeostasis and tolerance of self-antigens [9].

4. ROLE OF THE INNATE IMMUNE SYSTEM IN EPILEPSY

Activation of the innate immune system and the inflammatory changes within the central nervous system (CNS) that accompany it have been implicated in the development of seizures [10, 39]; however, the precise role of each element of the immune system currently remains unclear (Table **1**). Increasing evidence has shown that the innate immune system may react, not only to a systemic or intracerebral infection (bacterial or viral) but it also to brain injuries and seizure activity. Therefore, IIS activation and the inflammatory reactions in the brain that are associated with it may mediate some of the structural and molecular changes that occur in epileptogenesis and the long-term consequences of seizures [9, 10, 40].

Surgical resections of epileptogenic brain tissue from patients with chronic drug-resistant epilepsies have been observed to have important inflammatory processes [10]. These inflammatory processes were observed even in clinical cases of temporal lobe epilepsy and epilepsy-associated with malformations of cortical development, which typically do not have an inflammatory pathophysiology [41-43]. These findings, combined with clinical evidence that in some cases of drug-resistant epilepsies anti-inflammatory treatments can provide seizure control [10], strongly suggest there is an implication of inflammation of the brain with the development of seizures.

Research done with rodent models has shown that the Innate Immune system (astrocytes, microglia, the vasculature, and the BBB) plays a critical role in the cascade of molecular and cellular events contributing to seizures and epilepsy. Microglia are the major immune cells of the brain; they remove dying cells and cellular debris without inducing inflammation [44]. Activated microglia collect at the site of brain injury and secrete pro-inflammatory and/or anti-inflammatory cytokines in response to pathological insults such as infection or brain injury [45, 46]. Additionally, mounting evidence points to an important role for microglia in the regulation of aNS/PC under physiological conditions [47-49].

Although innate immunity in the brain is regulated primarily by microglia and astroglia, it is also mediated by neurons. It has been proposed that the SE and seizure activity leads to activation of these cells and the release of endogenous danger signals and cytokines causing a cascade of inflammatory events in other neurons and glia, *via* activation of their receptors [9, 34, 40, 50]. This process results in an increase of neuronal hyper-excitability and activation of the molecular chain of events involved in epileptogenesis [15, 51]. A vicious cycle is established in which the effects of inflammation of the brain contribute to the propagation of individual seizures and cell death, and these, then, promote further inflammation. This cycle of events culminates in the onset of spontaneous recurrent seizures (SRS) [15], (Fig. **1**).

4.1. Endogenous Danger Signals and Cytokines

Inflammatory mediators, such as cytokines regulate the activation of innate immunity and the transition to adaptive immunity. Cytokines are polypeptides that act as soluble mediators of inflammation and have a key role in in anti-inflammatory pathways. These molecules are crucial for

Table 1. Innate immune system receptors in epilepsy research.

Reference	Mediator	Seizure Model	Animal Model	Effect
[118]	IL-Iβ IL-IR1	IHpKA	Mice	Increased IL-Iβ expression in astrocytes and IL-IR1 expression in neurons and astrocytes after seizure.
[114]	TNF-α IL-1β	PTZ and 4-aminopyridine PILO	Rat	Seizure increased IL-1β and TNF-α expression. Anti-epileptic drugs reduced brain inflammation.
[119]	IL-1R1, IL-6 and TNF-R1	TMEV	Mice	IL-6 and TNF-α modulate signaling within the CNS and contribute to the development of seizures.
[110]	IL-1β, IL-1R1, TNF-α and IL-6	PTZ	Rat	Increased hippocampal expression of IL-1R1 during seizure development, but IL-1β expression only in fully kindled seizures.
[112]	IL-1β/IL-1R1 signaling	Electrical SE and PILO	Rat	IL-1β/IL-1 receptor blockade reduced cell loss after SE and provided neuroprotection.
[92]	TLR3	Poly(I:C)	Mice	TLR3 plays an important role in seizure susceptibility and epilepsy development.
[95]	TLR4 and RAGE	IHpKA	Mice	RAGE and TLR4 expression increased the duration and frequency of seizures.
[120]	TLR4	PILO	Mice	Increased TLR4 expression three days after PILO, but absence of TLR4 upregulation three weeks later, when GFAP levels were still increased.
[105]	TLR4	IHpKA	Mice	Increased TLR4 expression in neurons post-seizure and reduction of acute and chronic seizures by TLR4 antagonists.

CNS: Central nervous system; GFAP: Glial fibrillary acidic protein; IHp: Intrahippocampal; IL: Interleukin; IL-1R1: Interleukin-1 receptor type-1; KA: Kainic acid; PILO: Pilocarpine; Poly(I:C): Polyinosinic-polycytidylic acid; PTZ: Pentylenetetrazole; RAGE: Receptor for advanced glycation endproducts; SE: Status epilepticus; TLR: Toll-like receptor; TMEV: Theiler's murine encephalitis virus; TNF-R: Tumor necrosis factor receptor; TNF-α: Tumor necrosis factor alpha.

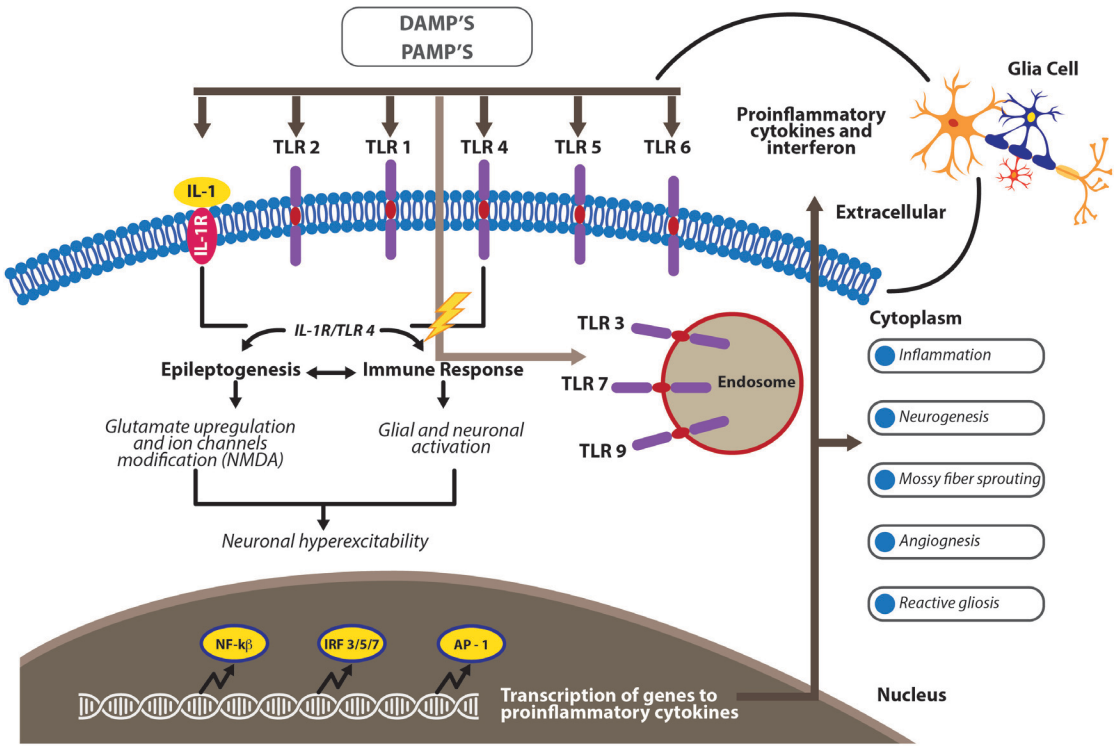

Fig. (1). Possible mechanism underlying the role of the innate immune system (IIS) in epilepsy. After an initial precipitating injury, a process is initiated in which the repetitive action of certain exogenous neuroactive substances can lead to major functional changes. Specifically, cytokines and danger signals induce inflammatory molecules responsible for direct activation of glial or neuronal signaling pathways, such as TLR4 and IL-1R by DAMPs or PAMPs. These inflammatory mediators can activate specific receptors that effect ion channels, upregulate glutamate signaling and elicit modifications that lead to increased neuronal excitability. The increased expression of pro-inflammatory cytokines and interferons has been reported to characterize the chronic epilepsy condition; this increase in expression may increase inflammation, contributing to the development of seizures. Transcriptional activation of genes can also be triggered by inflammatory molecules, which may perpetuate brain inflammation and contribute to long-term molecular plasticity involved in epileptogenesis. In addition, altered neurogenesis, neuronal cell death, increased mossy fiber sprouting and angiogenesis have been shown to be important factors in the recurrence of seizures and the development of epilepsy.

immune responses and combating infections; however, in the case of sepsis, inflammation, and trauma, they can become dysregulated and pathological [34, 37, 52]. Growth factors (such as transforming growth factor [TGF]-β), interferons (IFNs), interleukins (ILs) and tumor necrosis factors (TNFs) are all cytokine receptors.

Cytokines are produced by a broad range of immuno-competent cells including endothelial cells, as well as neurons and glia of the CNS. They enable communication between effector and target cells during a tissue injury or immune challenge. After their release, the cytokines interact with one or more of the cognate receptors. IL-1β, TNF, and IL-6 are the prototypical inflammatory cytokines of the CNS that have been most extensively studied [53-55].

The activity of cytokines can be regulated at multiple levels. These include cleavage of cytokine precursors (for example, pro-IL-1β, pro-TNF) by specific proteolytic enzymes, cellular release, and gene transcription. They can also be regulated by receptor signaling (see below). All brain cell types appear to be capable of releasing cytokines and their receptors. After CNS insult a low basal expression of these molecules is rapidly upregulated.

Chemokines are a specific class of cytokines that serve as chemoattractants that manage the migration of leukocytes from the bloodstream through the endothelial barrier into anatomical locations of inflammation, injury or homeostatic processes [56]. In addition to their involvement in the regulation of neural stem cell migration and microglial motility. These cytokines also provide axon guidance and promote angiogenesis neurogenesis, and synaptogenesis during development of the brain [57, 58]. Proinflammatory cytokines such as IL-1β often stimulate the release of chemokines. Cytokines have been reported to affect brain microvasculature (IL-1β can induce neovascularization) and to damage to the blood-brain barrier. These actions may be relevant for epilepsy as they lead to increased permeability that allows substances and cells to enter the brain that would otherwise be excluded.

4.2. IL-1 and Toll-Like Receptors

TLRs and Interleukin-1 receptors (IL-1) together form a receptor superfamily, called the "Il-1 / TLR superfamily"; all members of this family have a so-called TIR (toll-IL-1 receptor) domain in common. The TLRs consist of an extracellular N-terminal leucine-rich domain, a transmembrane domain and

an intracellular C-terminal signaling domain known as the TIR domain, which is homologous to the TIR domain of the IL-1R family [59].

TLRs are type I integral membrane glycoprotein receptors that play a key role in the innate immune system. The ability of the IIS to recognize conserved pathogen-associated molecular patterns (PAMPs) is due, in part, to the presence of these immune receptors. When TLR signaling is activated in immune cells such as macrophages and microglia, it initiates the generation of pro-inflammatory factors and promotes the elimination of pathogens. The TLR family includes 13 members (at least 10 in humans) [60]. A large range of these TLRs are expressed in cell cultures. However, this is partly due to the growth factors present in the medium or to cell preparation procedures that activate them mechanically. Both in rodents and in human tissue, the *in vivo* pattern of expression of the TLR receptors seems to be restricted for the most part to TLR2, TLR3, and TLR4 [61, 62].

TLR signaling comprises the recruitment of cytoplasmatic adaptor proteins; this induces protein kinase cascades which lead to activation of nuclear factor kB (NFκB) or interferon-γ-inducible gene activation and set in motion the inflammatory response [9]. When TLRs are activated by pathogens, it leads to cytokine release, for instance, IL-12. These cytokines are involved in the transition between innate and adaptive immunity [34].

4.3. Interleukins

IL-1R/TLR signaling is essential for the activation of IIS and inflammatory mediators. A series of studies in animal models of epilepsy, later corroborated by tissue specimens from patients with epilepsy, showed that seizures cause a dramatic proinflammatory signal mediated by microglia secreting cytokines such as interleukins. The IL-1R family contains nine members including the extracellular Ig-like domain and the intracellular Toll/interleukin receptor (TIR) domain [15]. *In vivo* evidence shows inherent but low expression of IL-1R1 or TLRs in brain cells; however, these receptors are upregulated in specific areas of the CNS in various pathological conditions, including seizures [61].

IL-1R/TLR signaling may occur either after recognition of pathogens, when IL-1β R recognizes binding proinflammatory molecules such as IL-1β or when TLRs that recognize danger signals such as HMGB1 are released from receptors that are activated on injured brain cells [15, 51]. IL-1R/TLR signaling is a homeostatic response of the brain that may cause pathological neuronal hyperexcitability exclusively when the duration or extent of signaling activation surpasses the homeostatic threshold [63].

IL-1R/TLRs initiate signaling cascades using at least two different pathways. The first is a myeloid differentiation factor 88 (MyD88)-dependent pathway that is common to all TLRs except TLR3. This adaptor protein pathway activates nuclear factor κβ which then triggers the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1 and IL-12. There is also a MyD88-independent pathway which is associated with dendritic cell maturation and the stimulation of IFN-β. TLR3 and TLR4 can signal through this pathway using a Toll/interleukin receptor domain-containing adaptor protein-inducing interferon β (TRIF)-dependent pathway. Type I IFNs acting on the TRIF-dependent pathway increase the expression of anti-inflammatory molecules, such as IFN-β [15, 64].

Interleukin-1β (IL-1β) has been shown to mediate several effects that are pro-convulsant, either indirectly or directly acting on neurons [65]. The binding of IL-1β to IL-1R1 and danger signal HMGB1 to TLR4 activate convergent signaling cascades [37, 66, 67]. When activated by these ligands, the downstream pathways merge with the TNF pathways at the transcription factor NFκB. NFκB regulates the synthesis of chemokines, cytokines, enzymes (such as COX-2) and TLRs, IL-1R1, and TNF p55 and p75 receptors [68]. The NFκB transcriptional pathway regulates the expression of genes that are implicated in cell survival and apoptosis, neurogenesis, and processes related to the molecular reorganization and plasticity of synapses [69]; all of which occur concomitantly with epileptogenesis in experimental models [70, 71].

The functional outcome of the activation of IL-1R/ TLR signaling in the brain is determined by the cell type that is expressing the receptors. Thus, the CNS immune response produces a variety of cellular responses, and although intracellular signaling in response to IL-1R1 or TLRs activation is highly convergent, there appears to be both cell and signal-specific diversity in the effector molecules that are produced and released. For instance, the expression of TLR2 and TLR4 in microglia have an autocrine role as they promote apoptosis of microglia after overactivation with PAMPs. It has also been reported extensively in the literature that microglia-expressed TLRs play a role in neuronal survival.

Thus, TLRs can either mediate soluble factor release from microglia, which has potentially damaging effects on neurons, or induce the phagocytic phenotype of microglia which can be neuroprotective. Moreover, similarly to T cells, TLRs have been shown to participate in the cross-talk between microglia and cells of adaptive immunity [72]. Studies in experimental models of ischemia suggest that when TLRs are expressed by neurons, they can contribute both to cell loss, [73] and Alzheimer's disease [74]. Also, as these receptors regulate neurogenesis and neurite outgrowth, they may contribute to neuronal plasticity [73, 74]. To give another example of a differential expression of these receptors, IL-1R1 may determine the activation of cell death or plasticity programs, and also effects the role of IL-1beta in physiological brain functions [75].

Activation of IL-1R1/TLR4 signaling may also trigger transcriptional changes. These changes could perpetuate inflammation *via* NFjB-dependent transcription of inflammatory genes, and as they promote the expression of genes that are involved in neurogenesis, cell death, and synaptic molecular reorganization and plasticity, they may also contribute to a chronic decrease in seizure threshold [76] (Fig. **1**). These changes occur during epileptogenesis and contribute to the transformation of healthy brain into tissue that generates seizures.

4.4. Innate Immune Receptors and Neurogenesis

The innate and adaptive immune systems are increasingly being considered as key modulators of hippocampal neurogenesis under both physiological and pathologic condi-

tions, including epilepsy [50]. Adult neurogenesis occurs in the normal adult brain and has been suggested to play a key role in the self-repair of neuronal networks in neuropathological conditions, as well as in the action of certain exogenous neuroactive substances. Chronic inflammation can affect synaptic plasticity, and the resulting alterations in adult neurogenesis and cell loss have been linked to the development of epilepsy.

Adult hippocampal neurogenesis refers to the continuous process of generation of new neurons throughout life. This process occurs *via* a population of proliferating neural stem/progenitor cells located in the subgranular zone of the adult hippocampal dentate gyrus (DG) [77]. Adult neurogenesis has been reported predominantly in two brain regions: in addition to the SGZ, it is found in the subventricular zone of the lateral ventricles [78, 79]. Under normal conditions, newly born dentate granular cells (DGCs) migrate a short distance into the granule cell layer (GCL) of the dentate gyrus, where they are incorporated into the preexisting neuronal network and acquire electrophysiological characteristics of mature DGCs [79, 80] (Fig. **2**). However, in the epileptic brain, this process is dramatically altered: the presence of ectopic cells in the hilus with abnormal dendritic and axonal reorganization has been described in both the acute and chronic stages of epilepsy.

Abnormal hippocampal adult neurogenesis is a prominent feature of animal models and patients with TLE [81, 82, 27]. Hippocampal cell changes such as alteration in postnatal neurogenesis, reorganization of neuronal circuitry and potentiation of synapses have been observed in various epilepsy models in response to damage, [82-86]. However, the evidence obtained in these studies is contradictory as to whether these changes are beneficial or detrimental (Fig. **2**). Recent literature has provided evidence of innate immune reactions that inhibit neurogenesis after initial brain insult (with a net effect of reducing onset, duration, and intensity of seizures). It has also been reported that the IIS contributes to the aberrant neurogenesis and network changes (increase in neural excitability) that lead to the onset of chronic epilepsy (Fig. **3**).

In 2014, Nunan *et al.* investigated the mechanisms that regulate neurogenesis. Their study demonstrated that survival and proliferation of neural stem/progenitor cells (NSPCs) was reduced by depleting microglia from hippocampal cultures. Moreover, when purified hippocampal microglia, or their conditioned media, were added to these cultures, it had a trophic and proliferative effect on the NSPCs. The prolifera-

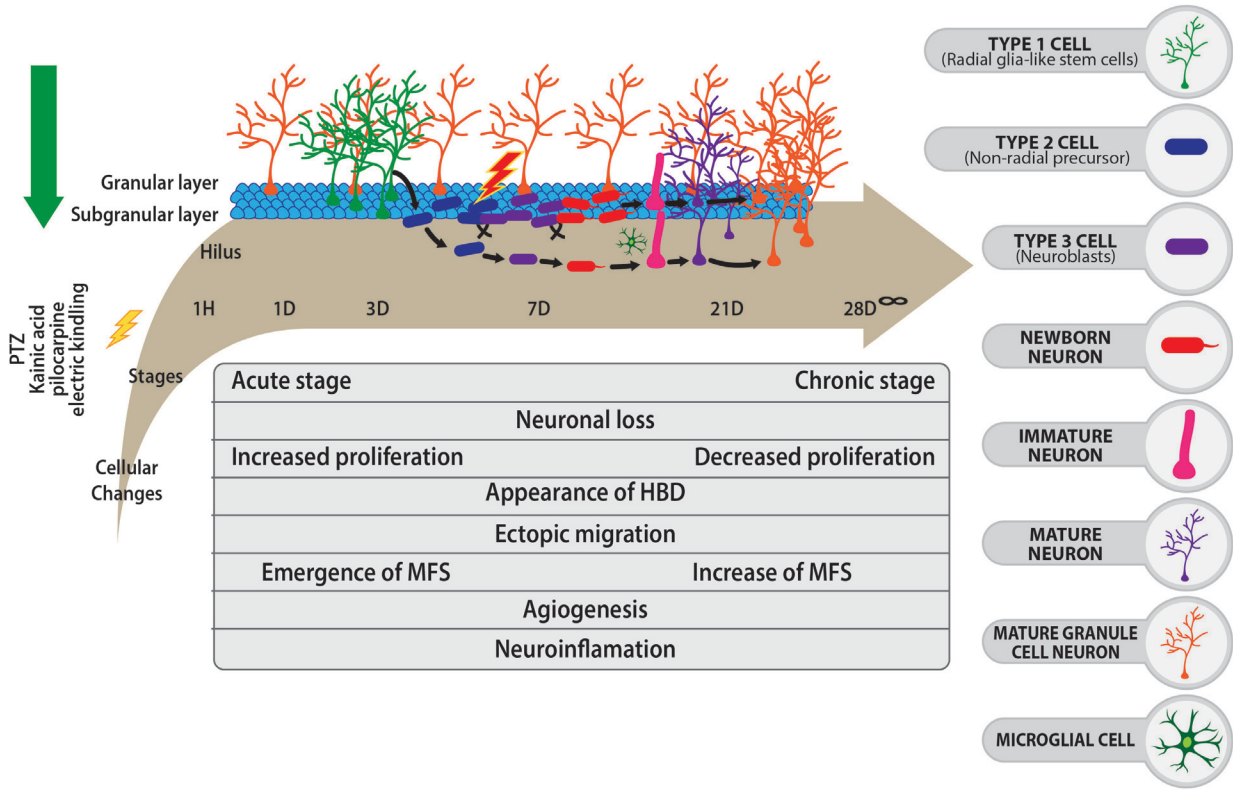

Fig. (2). Neurogenesis in the subgranular zone (SGZ) of the epileptic brain. Adult neurogenesis occurs in the normal adult brain and has been suggested to play a major role in the self-repair of neuronal networks in neuropathologic conditions, as well as in the action of certain exogenous neuroactive substances. Chronic inflammation can affect synaptic plasticity and the resulting alterations in adult neurogenesis, and cell loss have been linked to the development of epilepsy. In the healthy brain, new granule cells are generated from progenitors in the SGZ and typically integrate into the granular cell layer. However, in the epileptic brain, this process is dramatically altered: the presence of ectopic cells in the hilus with abnormal dendritic and axonal reorganization has been described in both the acute and chronic stages of epilepsy. In addition, neuronal loss, changes in the proliferation, emergence of hilar basal dendrites, angiogenesis, reactive gliosis (astrogliosis and microgliosis) and neuroinflammation are other pathophysiological features reported. Exactly how these elements contribute to the development of chronic seizures remains unclear.

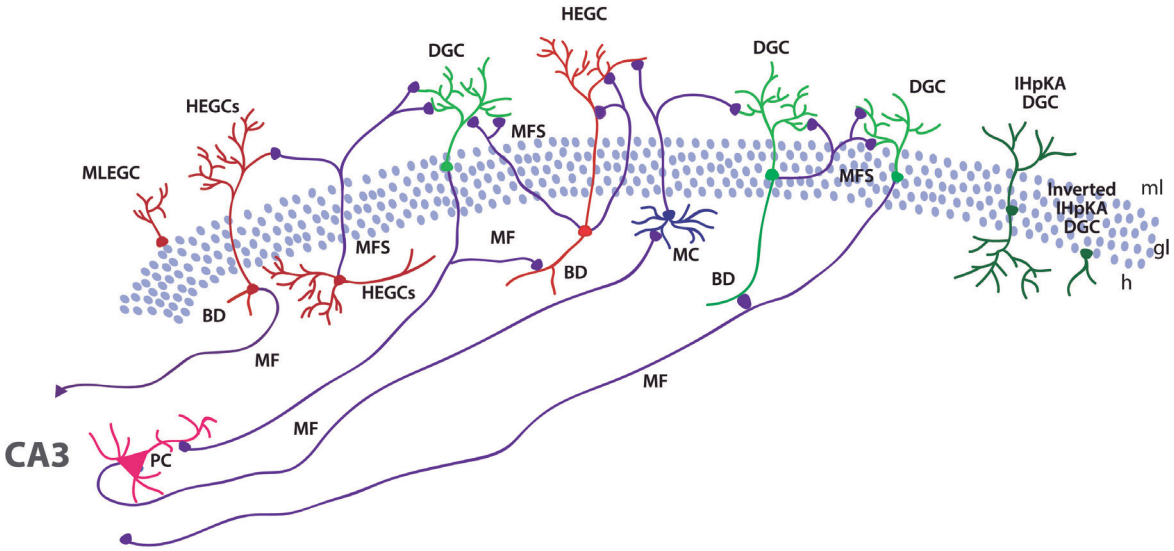

Fig. (3). Simplified scheme of diverse neurons found in the epileptic dentate gyrus and their excitatory connectivity. Adult hippocampal neurogenesis refers to the continuous process of generation of new neurons throughout life by a population of proliferating neural stem/progenitor cells located in the subgranular zone of the adult hippocampal dentate gyrus (DG). Under normal conditions, newly born dentate granular cells (DGCs) migrate a short distance into the granule cell layer (gl) of the dentate gyrus, where they are incorporated into the preexisting neuronal network and acquire electrophysiological characteristics of mature DGCs. Aberrant hippocampal adult neurogenesis is a prominent feature of TLE animal models. After status epilepticus, new dentate granule cells (DGCs) often show ectopic migration to molecular layer (ml) and dentate hilus (h). These cells may develop basal dendrites (BD) and mossy fiber sprouting (MFS). Recent literature has provided evidence of innate immune reactions that inhibit neurogenesis after initial brain insult (with a net effect of reducing seizures). Also reported are IIS contributions to the aberrant neurogenesis and network changes (increase in neural excitability) that lead to the onset of chronic epilepsy. Molecular layer ectopic granule cell (MLEGC); hilar ectopic granule cell (HEGC); mossy fiber axons (MF); mossy cells (MC), DGC induced by intra-hippocampal kainic acid (IHPKA).

tive and proneurogenic effect of microglia was enhanced by the release of vasointestinal polypeptide (VIP) *via* the VPAC1 receptor by dentate gyrus interneurons. This enhancement induced by the VIP was mediated by the release of IL-4, which acts directly on NSPCs. These findings show evidence of a potential immune-neurogenic pathway. Achieving disruption of this pathway may have significant implications for the treatment of conditions like epilepsy, where activation of the immune system, cognitive disability, and loss of interneurons, occur simultaneously [50].

Recently, inflammatory molecules (*e.g.*, IL-6) were reported to have an inhibitory action on neurogenesis [87]. Specifically, neuroinflammation induced by radiation injury or by lipopolysaccharide (LPS) was reported to inhibit neurogenesis. Also, an inflammatory blockade produced by indomethacin or inhibition of microglia activation with minocycline restored neurogenesis [39, 87, 88]. That neurogenesis is impaired by inflammation induced by TLR activation, represents a significant finding for research concerning the IIR regulation of neurogenesis. More recent studies have shown that in pathological conditions pro-inflammatory cytokines are induced by activated TLR signaling [77, 89, 90].

These IIS reactions are now considered to be important factors contributing both to the brain's initial attempts at recreating homeostasis and then the observed network changes that spiral into the chronic seizures and cognitive damage that characterize this disorder (Figs. **2** and **3**). Recent evidence suggests that innate immune receptors such as TLRs play a central role in chronic seizure development.

4.5. Innate Immune Receptors in epileptogenesis

TLR3 is primarily expressed intracellularly, where it acts as a sensor for double-stranded RNA and double-stranded mRNA, which is produced by replicating viruses, and induces high levels of type I IFNs, such as IFN-α and IFN-β [91]. Recently, a study involving agonist-induced activation of TLR3 in mouse models showed evidence of impairment of working memory and inhibition of neurogenesis [15]. It was reported that the synthetic TLR3 ligand, poly (I:C), promotes a sustained spontaneous activity in CA1 pyramidal cell layer of hippocampal slices. These results suggest that TLR3 have a modulator role in spontaneous excitability, affecting the expression of N-methyl-D-aspartate (NMDA) receptor subunit NR2B and astrocyte-specific glutamate/aspartate transporter (GLAST), responsible for homeostasis of glutamatergic neurotransmission in the hippocampus and the concentration of glutamate respectively. Both, NMDA and GLAST, play an important role in the modulation of excitability. So, in this way, TLR3 was shown, at least *in vitro,* to be involved in epileptogenesis [92] (Table **1**, Fig. **1**).

TLR4 is a transmembrane protein; its activation leads to an intracellular signaling pathway NF-kappaB and inflammatory cytokine production which in turn activates the innate immune system [93]. A recent study implicated TLR4 in the development and propagation of seizures in both humans and animal models [94]. Mice lacking functional TLR4 were *resistant* to seizures induced by proconvulsant chemicals. Furthermore, TLR4 antagonists decreased seizure frequency in wild-type animals with epilepsy. These observa-

tions suggest TLR4 has a pro-convulsant effect, which would imply that enhanced TLR4 activity may play a role in seizure genesis. Similarly, in a study by Iori *et al*., TLR4 knock-out mice treated with KA, showed an increased susceptibility to induced seizures, affecting their onset time and duration, suggesting again that TLR4 has a pro-ictiogenic effect [95]. In support of this, diverse data suggest that some receptors could act as neuromodulators, in fact, the activation of TLR4 and IL-1R1 through HMGB1 and IL-1β enhance the NMDA receptors mediated Ca2+ influx.

Although many studies have examined the role of TLR3 and TLR4 in epilepsy, the role of TLR2 signaling in the pathophysiology of the epileptic process is still under investigation. TLR2 is a membrane protein, a receptor expressed on the surface of certain cells that binds components of the Gram-positive bacterial cell walls. During neuroinflammation, it is also a marker of microglial activation [96]. A recent study employing a TLR2 agonist (LTA), and its antibody (LTA-A), investigated a potential role of TLR2 in the regulation of kindling epileptogenesis. A rapid kindling procedure was used on the animal models. When administered before kindling, LTA produced a reduction in the afterdischarge threshold. Subsequent administration of LTA-A, which acts as an immunological blockade of TLR2 lowered the frequency and severity of focal to bilateral tonic-clonic seizures. Their results suggest that TLR2 is involved in kindling epileptogenesis and in the increase in seizure susceptibility that kindling produces. They also showed that exogenous inhibition of TLR2 signaling reduces seizure related neuroinflammation in the hippocampus [97].

Toll-like receptor 9, is known to be a pathogen-sensing receptor for innate immune system activation. It recognizes self-DNA derived from degenerating neurons and induces TNF-a production in the microglia after seizures, which results in the inhibition of seizure-induced aberrant neurogenesis. As well as TLR3, TLR9 is located intracellularly in the endoplasmic reticulum and endosomes. TLR9 is also similar to TLR4, due is coupled to the signaling adapter MyD88, but TLR9 ligands include CpG oligodeoxynucleotides [98]. Both TLR9 and TLR4 display a similar cell type distribution; the two are expressed by multiple systemic immune cell types and by cells of the CNS [99, 100]. Several investigations have also suggested that TLR9 plays a protective role in the brain in ischemic models [101, 102]. This would support the hypothesis that the activation of TLR9 increases the expression of TNF-α and phosphoinositide 3-kinases. Both of these receptors are involved in regulating cellular activation, inflammatory responses, and apoptosis [101, 102]. It has even been suggested that TRL9 activation serves as preconditioning, so as to reduce neuronal damage [102].

Together these data indicate that TLR9 plays a neuroprotectant role, similarly to TLR2 and TLR4 [103]; however, the precise relationship between TLRs and epilepsy is still being explored. A work by Matsuda *et al.* offered new evidence that activation of TLR9 signaling in microglia by self-DNA derived from degenerating neurons induces the production of TNF-a. This was shown to suppress aberrant neurogenesis and reduce cognitive decline after seizure and recurrent seizure severity [77]. They conclude that bidirectional communication exists between the innate immune and nervous systems for homeostatic neurogenesis in the adult hippocampus [77].

However, this hypothesis was challenged in a study by a group from Italy [104]. Their study states that microglia and myeloid cells such as macrophages have always been difficult to distinguish due to an overlap in expressed cell surface molecules. Thus, the detrimental role in epilepsy that is attributed to microglia might be shared with myeloid infiltrates. They observed that microglia does not express MHCII whereas myeloid infiltrates show high levels of MHCII and CD40 96 h after SE. Moreover, microglia only showed TNFα 24 h after SE while myeloid infiltrates revealed elevated levels of IL-1β and TNFα. Finally, both cell types showed the phagocytosis receptor Axl, pointing to phagocytosis of apoptotic cells as one of the primary functions of microglia. Their data suggest that, during early epileptogenesis, microglia from the hippocampus remain rather immune suppressed whereas myeloid infiltrates display an active inflammatory profile.

These results suggest that increased expression of immunological receptors might also contribute to neuronal hyperexcitability [15, 94, 105]. Although further evidence is needed to understand the link between endogenous TLR ligands and the CNS, studies suggest that immunological receptors such as TLR4 and TLR2 may contribute to the generation and perpetuation of seizures [94, 95, 105].

4.6. Innate Immune Receptors in Chronic Epilepsy

Immunological receptors such as receptor for advanced glycation end products (RAGE) are expressed under normal and pathological conditions in the pyramidal neurons, granule cells, and hilar interneurons of human and rodent tissues. In epileptic brain tissue, RAGE is also expressed in reactive astrocytes and blood vessels; this receptor is reported to have ictogenic properties [95]. This study suggests that the expression of immunological receptors in blood vessels may have an effect on the BBB, compromising SNC integrity and promoting aberrant neuronal activity through external factors such as peripheral mononuclear phagocytes.

As with RAGE, the expression of TLR4 was observed in pyramidal neurons and reactive astrocytes (Maroso *et al.*, 2010), but was absent in microglial cells (Pernhorst *et al.*, 2013). The expression of TLR4 in chronic epileptic tissue is correlated with seizure frequency (Pernhorst *et al.*, 2013). Also, the interaction of TLR4 with endogenous ligands secreted by microglial and astrocytes, such as HMGB1, has been reported in brain tissue in the chronic stage of epilepsy. This suggests that their interaction enhances the expression of proconvulsant inflammatory molecules (TNF-α and IL-1β) in astroglia and microglia and promotes calcium flux through NMDA channels. Study of this mechanism is interesting as it could lead to the discovery of new therapeutic options to reduce epileptogenic activity [94, 106].

In addition, recent evidence demonstrates that TLR4 activation promotes the expression of IL-1β and that this cytokine suppresses GABAergic synaptic activity (presynaptic and postsynaptic) [107]. This abnormal GABAergic function is observed in many models of epilepsy [108]. Thus, this

evidence proposes that dysregulation of TLR4 is behind epilepsy; inflammatory processes caused by seizures and pro- and anti-inflammatory signaling converge as a physiological response, which contributes to epileptogenesis and the subsequent perpetuation of chronic epilepsy.

Several mechanisms of the IIS have been identified that attenuate the inflammatory responses, indicating the importance of such strict controls for homeostasis and prevention of injury [9].

4.7. Inflammatory Molecules in Epileptogenesis

The release of cytokines is one of the possible mechanisms underlying the role of inflammation processes in epileptogenesis [105]. Experimental models have demonstrated that, following SE, the expression of pro-inflammatory molecules such as interleukin (IL)-1β, IL-6 and TNF-α is increased, mainly in the parietal cortex, hippocampus and amygdala. Even, the IL-1β and TNF-α seem to play an important role upregulation of GABA transporters 1 and 3 (GAT-1 and GAT-3) expressed in neurons and glial cells respectively [109]. These carriers are essential to extracellular GABA regulation.

Since increased expression of GAT-1 and GAT-3 decrease inhibitory effects through GABA, these pro-inflammatory molecules might contribute to neuronal excitability, seizure susceptibility and epileptogenesis [109]. However, other evidence shows that during the epileptogenic period the hippocampal expression of TNF-α does not change, therefore, the expression of this proinflammatory molecule is controversial [110, 111]. Furthermore, it is important to consider that observed effects may depend on which chemoconvulsant is used [109-111].

Similarly, experimental results differ regarding the hippocampal expression of IL-6 and IL-10; this was reported to increase after SE, with no change of expression in the parietal cortex [111], while other evidence showed that no important changes occur in hippocampal levels of IL-6 immediately after SE and during kindling process [110]. Also, no changes were reported in the hippocampal expression of cytokines such as IL-2, IL-4, IFN-γ, and IL-17, after SE [111]. Therefore, two hypotheses may be considered; one is the possibility that cytokines have a neuroprotective effect after injury, or they trigger neuronal excitability that increases the brain's vulnerability to seizure.

Likewise, during the kindling process, the expression of IL-1β was less than in the control group whereas its expression increased in fully kindled animals, these observations suggest that IL-1β plays an important role in the progress of hyperexcitability during kindling and in ictal activity spread [110, 112]. This is particularly important because evidence suggests that the receptor IL-1R1 is constitutively expressed in hippocampal pyramidal neurons under physiological conditions, while astrocytes and microglia are only present after injury [113]. Surprisingly, the expression of the IL-1R1 was increased in the dentate gyrus both when fully kindled and during kindling process [110].

Moreover, antiepileptic drugs have been demonstrated in rat hippocampus to decrease the expression of IL-1β and TNF-α [114, 115]. In fact, a recent study also showed in two different SE models that the IL-1β/IL-1R blockade with anti-inflammatory drugs exhibited a significantly decreased expression of IL-1β in neurons and astrocytes of CA1, CA3, and of the entorhinal cortex. This suggests that the use of anti-inflammatory drugs could have a neuroprotective role [112].

4.8. Inflammatory Molecules in Chronic Epilepsy

The exact mechanisms that underlie the role of the innate IIS in epilepsy are still unknown. However, the activation of certain inflammatory receptors, such as TLR4 and IL-1R, by endogenous and exogenous molecules, is associated with the development of this disorder. Once activated, the inflammatory receptors trigger signaling cascades that promote the secretion of proinflammatory cytokines and IFNs that in turn, favor the immunological processes as well as inflammation and glial activation. Furthermore, it is possible that the immunological receptors modify the regulation of NMDA and GABA receptors generating a neuronal hyperexcitability which in turn increases the immune response creating a vicious cycle (Fig. **1**).

Additionally, epileptic brain slices show that IL-1β increases the excitability of CA1 hippocampal neurons by decreasing the amplitude of NMDA-induced outward currents and increasing the magnitude of NMDA-induced inward currents [116]. Despite this, no demonstrable effects were seen on the onset of SRS or the frequency or duration of seizures in rats with chronic epilepsy that were treated with an IL-1R antagonist and inhibitor of IL-1β; but the use of this inflammatory treatment before the SE showed neuroprotective effects [112]. The above findings suggest that the use of anti-inflammatory molecules in conjunction with antiepileptic treatment might improve the prognosis of this neurological disorder. However, it is unclear if the use of anti-inflammatory molecules is beneficial or harmful, especially as it has been reported that the pharmacological inhibition of the microglial activation or TNF-α production results in exacerbated aberrant neurogenesis [77], increasing the abnormal neuronal connectivity and the subsequent injury.

Evidence is mounting that the changes to both hippocampal neurons and IIS receptors, such as TLRs, are involved in epileptogenesis and have a role in the development of chronic seizures. This highlights the possibility that targeting these receptors in epilepsy research may provide possible avenues for epilepsy treatments that could diminish the cognitive impairment associated with this disorder.

CONCLUSION

The present review suggests that there is an interesting relationship between the innate immune receptors and the development of epilepsy (Table **1** and Fig. **1**). The reviewed literature proposes that the innate immune system plays a role in the development of epilepsy by helping to create an environment, in which the brain is more susceptible to aberrant neuronal activity, *i.e.* seizure (Figs. **1** and **2**). Although evidence points to an implication between innate immune responses and epileptogenesis, the functional significance of these and other changes such as the alterations in neurogenesis, remain unclear.

There are currently no surgical or medical interventions that will prevent the onset of epilepsy. At present, therapeutic options are very limited. Epilepsy seizures can be difficult to control; nearly half of the patients with this condition fail to respond to anticonvulsants. The available medications for epilepsy only alleviate its symptoms, and this leaves patients and their families in dire need of new treatment approaches. Our limited understanding of the molecular underpinnings of this disorder makes it imperative to conduct research that targets the disease mechanisms underlying the predisposition to recurrent seizures and to discover treatment protocols that intervene early enough in the process of epilepsy maturation to prevent the establishment of chronic epilepsy. For example, attenuation of seizure-induced inflammatory response by the exogenous inhibition of TLR2 with LTA-A is an interesting finding that may lead to a treatment that will impede epileptogenesis.

Research is currently underway to find disease-modifying therapies. As antagonism of the interleukin-1β receptor has an anticonvulsant effect, it has been suggested as a new target for potential anti-seizure drugs [13]. The identification of endogenous ligands is considered to be another promising avenue for epilepsy research. Findings linking TLR's with aberrant neurogenesis in epilepsy indicate that a promising concept for future therapeutics for the cognitive disabilities in epilepsy might be a modulation of these receptors. Other vectors of interest for future studies are the inhibition of seizure-induced neurogenesis during early phase and the stimulation of neurogenesis in the chronic phase [117, 27]. Scientists hope that modifying seizure-induced neurogenesis would both curb its harmful effects and assist the ones that are neuroprotective and regenerative. To this end, several ways of manipulating (promoting or inhibiting) adult neurogenesis *in vivo* are presently under development [118-120].

Furthermore, the mechanisms and clinical implications of the epilepsy-related immune alterations need to be clarified. Studies that combine the IIS, brain cellular changes and neurogenesis in an epilepsy animal model could explain these interactions and lead to the development of new treatment strategies. Novel methodologies, such as the use of immunologically altered animals, are being used to study these interactions, and should help to clarify the role of the immune system in epilepsy. Future research that eventually explains the mechanisms underlying this disorder will contribute significantly to the development of better anti-epileptogenic therapies.

LIST OF ABREVIATIONS

APCs = Antigen-presenting Cells
BBB = Brain Blood Barrier
GABA = γ-aminobutyric Acid
NSPCs = Neural Stem/Progenitor Cells
PILO = Pilocarpine
PTZ = Pentylenetetrazol
RAGE = Receptor for Advanced Glycation end Products
SE = Status Epilepticus
SRS = Spontaneous Recurrent Seizures
TIR = Toll/Interleukin Receptor
TLE = Temporal Lobe Epilepsy
TLRs = Toll-like Receptors

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Toxicology

Apoptosis in pancreatic β-cells is induced by arsenic and atorvastatin in Wistar rats with diabetes mellitus type 2

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ABSTRACT

Introduction: Diabetes Mellitus type 2 (T2D) is a multifactorial disease. However, it is known that there is an important effect in pancreatic β-cells caused by apoptosis of pro-apoptotic proteins, possibly related to arsenic exposure and atorvastatin treatment.
Objective: The goal of this study was to evaluate the effects of atorvastatin treatment on apoptosis of pancreatic β-cells in Wistar rats with induced diabetes type 2 exposed to arsenic.
Material & methods: T2D in Wistar rats was induced by administration of Streptozotocin. The plasmatic glucose concentrations were measured using the glucose oxidase method, and the concentration of glycated hemoglobin (HbA1c) in whole blood was determined. Exposure to arsenic was measured from urine using atomic absorption with hydride generation, and pro-apoptotic proteins in pancreatic β-cells were observed using the Western blotting technique.
Results: Caspase-3 was present in rats that were treated with 10 mg/kg of oral atorvastatin and exposed to 0.01 and 0.025 mg/L of arsenic, but no others proteins were present, such as pro Caspase-8, bcl-2, and Fas. The glycemic levels were 129.2 ± 7.0 mg/dL in the control group and 161.8 ± 14.6 mg/dL and 198.3 ± 18.2 mg/dL ($p < .05$) in the study groups. HbA1c increased from 2.53% to 3.64% ($p < .05$) in the control and study groups.
Conclusions: Atorvastatin treatment and arsenic exposure alone are capable of generating apoptosis in pancreatic β-cells of Wistar rats with T2D. Together, all of these factors induce apoptosis in pancreatic cells.

1. Introduction

Diabetes is a serious, chronic disease that occurs either when the pancreas does not produce enough insulin (a hormone that regulates blood glucose) or when the body cannot effectively use the insulin that it produces [1]. Globally, an estimated 422 million adults were living with diabetes in 2014 compared to 108 million in 1980. The global prevalence (age-standardized) of diabetes has nearly doubled since 1980, rising from 4.7% to 8.5% of the adult population [1]. The most common form of diabetes mellitus is type 2 diabetes mellitus (T2D), which affects approximately 400 million people and imposes a substantial public health burden owing to a range of diabetic complications and the inadequacy of current therapies [2]. T2D presents itself as chronic hyperglycemia and is thought to result from the progressive failure of pancreatic β cells in which they do not secrete sufficient insulin to meet metabolic demands [2].

Pathogenesis of T2D is predisposed by genetically abnormal islets. This abnormality may be a reduced islet cell mass or accelerated apoptosis. Some individuals who remain lean and fit may never develop diabetes or may do so at a very old age because of progressive

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deterioration of cell function. Others become susceptible because of weight gain, physical inactivity, high-fat diets, medications and other associated lifestyle factors such as environmental contaminants. These include, for example, inorganic arsenic (iAs) exposure, which causes a risk of developing T2D [3,4].

Most drugs that are used to treat T2D are primarily intended to lower blood glucose levels, but have, thus far, been unable to change the progressive course of T2D or adequately reduce the risk of late-stage complications associated with diabetes mellitus [5]. Previous studies have reported that therapy with anti-hyper lipidemia drugs (including atorvastatin and simvastatin) is accompanied by an increased risk of type 2 diabetes through decreases in insulin secretion and insulin sensitivity [6,7]. The influence of statins on pancreatic β-cells lines and cytotoxic effects vary and should be carefully estimated by various cell lines in different conditions [8]. However, Sadighara M et al. proposed that the toxicity of atorvastatin to pancreas mitochondria is a key point of drug-induced apoptotic cell loss in the pancreas and is therefore a justification for the increased risk of diabetes mellitus [9]. Other studies performed by Kuoppala J et al. and Thisted H et al. conclude that Statin use seems to be associated with an increased risk of acute pancreatitis. The association is more apparent during the first year of statin use and among former users and may even indicate a mild protective effect [10,11].

Alternatively, the environmental exposure that drives the development of T2D and obesity must effective, given the rapid shifts in disease prevalence that they have engendered, and pervasive, given that no contemporary industrialized population has been spared. Epidemiological studies have highlighted many potential environmental “perpetrators,” the combination of physical inactivity and caloric excess being the most prominent [12]. There are, however, many other plausible environmental factors for which a role has been advanced, including arsenic (As) that is present in drinking water and is contacted from work exposure [13]. Navas-Acien et al. demonstrated a positive association between total urine arsenic, likely reflecting inorganic As exposure from drinking water and food, with the prevalence of type 2 diabetes in a population that had low to moderate As exposure [14]. Additionally, Mendez MA et al. suggested that there is potential risk of T2D associated with chronic exposure to inorganic arsenic (iAs) at levels < 0.10 g/L in drinking water in a northern Mexican population [15].

Reference to apoptosis

There is a lack of detailed information about how As and treatment with statins could lead to diabetes because it is a very complex problem. In this paper, we studied the effects of atorvastatin and As exposure on apoptosis in the pancreatic β-cells in Wistar rats with induced T2D.

2. Materials and methods

2.1. Animals

The experimental protocols were approved by the Institutional Animal Ethics Committee of Juarez University of Durango State and followed the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals. Newborn Wistar Kyoto rats were randomly divided into eight different experimental groups with six animals in a group (three females and three males), were isolated and were marked with a tag for identification. The rats were obtained from the laboratory animal facility of Chemistry Faculty member Gomez Palacio of the Juarez University of Durango State, Dgo., Mexico. For the realization of this study, all laboratory animals were treated and handled in accordance with the recommendations put forth by the guide for the care and use of laboratory animals, published by the National Institute of Health [16].

2.2. Experimental design

The Wistar Kyoto rats were divided int0 eight experimental groups (G1 Healthy rats; G2 T2D rats; G3 T2D Arsenic (0.025 mg/L) exposed rats; G4 T2D + Arsenic (0.01 mg/L) exposed rats; G5 Healthy + atorvastatin (10 mg/kg) exposed rats; G6 T2D + atorvastatin (10 mg/kg) exposed rats; G7 T2D + arsenic (0.025 mg/L) + atorvastatin (10 mg/kg) exposed rats; G8 T2D + arsenic (0.01 mg/L) + atorvastatin (10 mg/kg) exposed rats. The experimental groups were observed for six months until the study was finished.

2.3. Reagent preparation

Streptozotocin (STZ) reagent preparation: A 0.01 M citrate buffer solution was prepared (dissolved 147 mg of citrate to 50 mL saline solution and pH adjust to 5.4). 200 mg STZ were diluted in 10 mL of 0.01 M citrate buffer solution to a final concentration of 20 mg/mL = 0.1 mg/5 µL. Atorvastatin calcium salt trihydrate was diluted with 10 mg/mL dimethyl sulfoxide. Sodium meta-arsenite was diluted in distilled and deionized water to achieve final iAs concentrations of 0.01 and 0.025 mg/L. All reagents were acquired from the Sigma-Aldrich Co. LLC. (St Louis, MO 63103, USA).

2.4. T2D induction with STZ

Type 2 diabetes was induced in rats 24 h after birth by intra-peritoneal administration of 100 mg/kg life-weight of STZ. The animals were housed in a petri dish cage in a ventilated room (one animal/cage) with a controlled temperature and artificial lighting conditions. Food and water were supplied ad libitum. A 12 h light/dark cycle was maintained until the rats were 250 g in weight.

2.5. Arsenic exposition

Rats groups G3 and G7 were exposed to 0.025 mg/L iAs concentration, and groups G4 and G8 were exposed to 0.010 mg/L iAs concentration. These groups were exposed through daily water intake for a six-month period.

2.6. Atorvastatin exposure

Groups G5, G6, G7, and G8 were exposed to two doses of Atorvastatin calcium salt trihydrate (10 mg/kg) through a nasogastric tube at 2.5 and 5 months-old during the experiment.

2.7. Glucose and HbA1c determinations

During the experiment, in two-month intervals, 2 mL of whole blood was collected from the rats through the caudal artery puncture. Plasmatic glucose in serum and Hb1Ac in whole blood were determined using the glucose oxidase and immuno-chromatographic methods, respectively.

2.8. Inorganic arsenic determination

During the experiment, urine samples were collected and iAs concentrations were determined using atomic absorption spectroscopy with hydride generation.

2.9. Recollection of tissue and pancreatic β-cells

After the study, pancreatic tissue was obtained through an abdominal incision. Once obtained, the pancreas was placed in a sterile container. The tissue was macerated with 5 mL of cold phosphate buffer solution (PBS 1x). Later, pancreatic cells were recovered by

centrifugation at 263*g*, 5 min at 4 °C and decanted. Cells were re-suspended in 1 mL of cold PBS 1x and were counted in a Neubauer camera. Later, cells were centrifuged at 263*g* for 10 min, decanted and pellet harvested, and immediately frozen in dry ice and ethanol for 5–10 s. The cells were re-suspended in 100 µL hypotonic buffer to obtain cytoplasmic proteins by centrifugation at 1463 g for 4 min at 4 °C. The supernatant fraction that corresponded to cytoplasmic proteins was stored at −70 °C until experiments were performed.

2.10. Electrophoresis and protein quantitation

To determine protein concentrations in the plasmatic fraction, the Bradford method [17] was used and Bovine albumin serum was used as a standard concentration. The cytoplasmic proteins were separated by vertical electrophoresis in 10% polyacrylamide gels under denaturing conditions (SDS-PAGE) [18]. The proteins were transferred to nitrocellulose films using transference equipment for 15 min at 15 V.

2.11. Western-Blot analysis

To perform Western-Blot analysis, a BioRad Opti-4CN kit and a peroxidase colorimetric substrate consisting in 4-cloro-1-naphthol (4CN) was used. Once the proteins were transferred to the nitrocellulose membrane, the Western-Blot analysis was performed using caspase-3 (H-277): sc-7148, IgG polyclonal rabbit antibodies; Caspase-8 p18 (H-34): sc7890, IgG polyclonal rabbit antibodies; FAS (FL-335): sc-7886 polyclonal rabbit antibodies and Bcl2 (C-2): sc-7382, polyclonal mouse antibodies. All antibodies were provided by Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). For incubation of primary antibody, a hypotonic phosphate buffer saline (HPBS) was eliminated and antibodies (anti-caspase-3, anti-caspase-8, anti-Bcl-2 and anti-FAS) were diluted to 1:1000 and were incubated for 2 h with agitation at room temperature. Later, using Phosphate Buffered Saline with Tween 20 (PBST), the cells were washed two times for 5 min and slowly agitated to eliminate the primary antibody. The secondary antibody (rabbit or mouse anti-IgG) was then incubated. First, PBST was eliminated and a dilution of 1:1000 was prepared and incubated for 2 h with agitation at room temperature. Later, using PBST, the cells were washed two times for 2 h and slow agitated to eliminate the secondary antibody.

Amplification was performed by adding 1x BAR solution. After incubation for 10 min and slow agitation at room temperature, the 1x BAR solution was eliminated. The membrane was washed four times for 5 min with 20% PBST/Dimethyl sulfoxide. Later, a membrane was prepared with 85 µL of streptavidin-HRP dilute on a 1:1000/centimeter-square. The membrane was incubated for 30 min and was slowly agitated with streptavidin-HRP. The substrate was diluted afterward and was eliminated, and the membrane was washed three times for 5 min and was slowly agitated with distilled water. Membrane photography was obtained from a photo-document management system.

3. Results

Over the course of the study, levels of glucose in the Wistar rats were determined based on exposure to STZ (induced T2D model). The G2 group showed glucose values (172.5 ± 27.9 mg/dL) during the six months of treatment, which were greater than those in the G1 group (129.2 ± 6.1 mg/dL) and HbA1c values (3.84 ± 0.6% vs. 3.16 ± 0.5%), respectively. Similar concentrations were observed for groups with induced T2D and other interventions (G3, G4, G5, G6, G7, and G8). Inorganic arsenic levels found in the urine of the different study groups (Table 1) showed the following: The G3 and G7 groups showed a range of 0.004–0.061 mg/L of arsenic (using 0.025 mg/dL as exposition concentration). The G4 and G8 groups showed a range 0.0013–0.043 mg/L of arsenic, similar to the exposure concentration (0.010 mg/L). Arsenic levels in groups G1, G2, G5 and G6 were undetectable (0.00 mg/L).

After performing the analyses for glucose and HbA1c parameters, it is apparent that groups G2, G3, G4, G5, G6, and G7 show a statistically significant increase ($p < .05$). The G5 group presented the smallest change (129.2 ± 7.0 mg/dL vs. 161.9 ± 14.6 mg/dL) and the G7 group presented the largest change (198.3 ± 18.2 mg/dL vs. 129.2 ± 7.0 mg/dL). On the other hand, after analyzing the Hb1Ac values, it was found that only groups G3, G4, G6, and G7 showed a statistically significant increase ($p < .05$). With respect to the healthy control group (G1), the values ranged from 2.53% (G7 vs. G1) as the lowest value and 3.66% as the highest value (G6 vs. G1) (Table 2). This was related to protein analysis. The analysis showed that pro-apoptotic protein caspase-3 was present in groups G4, G5, G6, G7, and G8. However, caspase-8 was not observed in any group. Procaspase-8 was positive in groups G1, G2, G4 and G6. Bcl-2 protein was present in groups G1, G2, G4, and G6, whereas FAS protein was present in groups G1, G2, G4, G6, G7 and G8 (Table 3). These data are demonstrated by the presence of bands on polyacrylamide gel (Fig. 1).

4. Discussion

The term “diabetes mellitus” describes a metabolic disorder of multiple etiology that is characterized by chronic hyperglycemia with disturbances to carbohydrate, fat and protein metabolism that result from defects in insulin secretion, insulin action, or a combination of both. Effects of diabetes mellitus include long-term damage, dysfunction, and failure of various organs [19]. Nonhuman models (NHM) have been widely used to study the fundamental aspects of T2D. Induction of T2D in NHM by injection of the ß-cell toxic drug (STZ) or total pancreatectomy is well-documented in the literature [20,21]. “Streptozotocin diabetes” is caused by the specific necrosis of the pancreatic ??-cells, and this agent is the first choice for diabetes induction in animals [22]. The model of neonatal administration of STZ (100 mg/kg) 48 h after birth is a method in which the pancreas undergoes partial destruction and regeneration, resulting in an animal with moderate hyperglycemia of 150–180 mg/dL [23–25]. This induction model has been used by several authors with diverse doses and administration times of STZ [26,27].

In this study, the STZ (100 mg/kg) was administered to Wistar rats 24 h after birth by intraperitoneal methods. The results showed serum glucose levels that were in agreement with proposed goals of T2D for groups G2, G3, G4, G6, G7 and G8 and were higher than the glucose and HbA1c levels of the healthy control group. These conditions were maintained for the six months of the study. Likewise, the only group that did not attain serum glucose levels to achieve a significant change was group G8, which was probably due to a reversal of the damage in the pancreatic cells and the possible adaptation the model to damage by STZ after administration. This effect has been reported by other authors [28–30]. Other causes of T2D development, such as prolonged arsenic exposure through drinking water, are important factors that cause the high prevalence and incidence of T2D [31–33]. In Mexico, Del Razo Luz M et al. reported a link between exposure to iAs in drinking water and an increased risk of developing diabetes, which is characterized by fasting hyperglycemia and impaired glucose tolerance. However, unlike typical type-2 diabetes, iAs-related diabetes was not associated in this study with increased insulin resistance as measured by HOMA-IR. In addition, low fasting plasma insulin levels seem to suggest that β-cell function may be impaired by exposure to iAs or that it is toxic [34]. To understand the effects of iAS on T2D, different studies with animal models have been performed [35]. A study using animal models suggested that high doses of iAs lowers the viability of β-cells and decreases its dose-dependent functional capacity [36]. Recent publications suggest that masses of β-cells in the pancreas can be lowered in T2D patients when there is disequilibrium between cellular regeneration and apoptosis [37,38]. In this study, a comparison between pancreatic tissues in healthy vs. T2D rats was performed, and the rats with

Table 1
Inorganic arsenic levels in the urine of the different rat groups.

Groups (6-animals)	Characteristic of groups	Mean concentration iAs (mg/L)	Standard deviation	Min/Max iAs concentration
G1	Healthy	0.0057	0.0018	0.002/0.007
G2	T2D	0.0027	0.0017	0.000/0.004
G3	T2D + 0.025 mg/L iAS	0.0424	0.018	0.013/0.061
G4	T2D + 0.010 mg/L iAs	0.0305	0.011	0.013/0.043
G5	Healthy + Atorvastatin	0.0026	0.003	0.003/0.009
G6	T2D + Atorvastatin	0.0025	0.001	0.000/0.006
G7	T2D + Atorvastatin + 0.025 mg/L iAs	0.0586	0.024	0.015/0.063
G8	T2D + Atorvastatin + 0.010 mg/L iAs	0.0393	0.004	0.021/0.059

Table 2
Glucose concentrations and HbA1c in the different experimental groups.

Group	Glucose (mg/dL) media ± SD	HbA1c (%) media ± SD
G1	129.245 ± 7.037	2.575 ± 0.180
G2	184.997 ± 24.930*	4.078 ± 0.673
G3	165.671 ± 13.762*	5.583 ± 1.083*
G4	190.583 ± 22.295*	5.367 ± 1.244*
G5	161.889 ± 14.615*	3.511 ± 0.142
G6	172.317 ± 13.553*	6.239 ± 1.595*
G7	198.317 ± 18.230*	5.106 ± 1.144*
G8	161.839 ± 9.680	0.394 ± 0.227

Values are presented as media of four determinations (one each 2 months).
*Represent values with a difference statistically significative ($p < .05$) respect to G1 control group.

Table 3
Results of Western-blot for pro-apoptotic proteins Caspase-3, caspase-8 and Fas and, anti-apoptotic protein Bcl-2.

Group	Caspase-3	Procaspase-8	Caspase-8	Bcl-2	Fas
G1	−	+	−	+/−	+
G2	−	+	−	+	+
G3	−	−	−	−	−
G4	+	+	−	+/−	+
G5	+	−	−	−	−
G6	+	+	−	+	+
G7	+	−	−	−	+
G8	+	−	−	−	+

(+) indicate presence, (−) Indicate absence and (+/−) indicate irregular presence.

T2D presented a decrease in pancreatic masses and hypocellular morphology. There are several potential mechanisms for β-cell dysfunction in type 2 diabetes, including the toxic effects of elevated glucose and/or lipid levels, effects of increased secretory demand because of insulin resistance, amyloid deposition, and the effects of altered levels of cytokines/adipokines [39]. Associated mechanisms of early β-cell demise include mitochondrial dysfunction, oxidative stress, ER stress, dysfunctional triglyceride/free fatty acid cycling, and glucolipotoxicity. Once hyperglycemia develops, additional processes are linked to glucotoxicity and the diabetic milieu, such as islet inflammation, O-linked glycosylation, and amyloid deposition. These accelerate β-cell demise, resulting in severe β-cell phenotypic alterations and loss of β-cell mass by apoptosis [40]. In this research, we demonstrated the activation of caspase-3 in the G4 group, but not in groups G1 or G2. This may indicate that iAS exposure (0.010 mg/dL concentration) for six months activates caspase, inducting pancreatic β-cell apoptosis. With respect to groups G5, G6, and G8, there were low concentrations of caspase-3, but with different patterns of intensity between groups, with the highest in group 6 (Table 3 & Fig. 1). Knowing that these groups were treated with atorvastatin, it is possible that the drug induced apoptosis and cell death, activating the caspase-3 pathway. This is because in the G5 group (healthy rats treated with atorvastatin), it was observed that the caspase-3 activity (compared with the G1 group; control group) did not exist. So, atorvastatin induces apoptosis by activating the caspase-3 pathway. In addition, the G6 group showed the most intensity in the blot analysis, indicating an increase in caspase-3 activity due to the presence of T2D and atorvastatin. Additionally, a lack of caspase-3 activity in the G2 group (rats with T2D but without atorvastatin treatment) supports this (Table 3 & Fig. 1). Groups G7 and G8 present a blot analysis that shows caspase-3 activity, but there is only minor intensity for the G6 group, and the G6 group has a higher blot intensity than the G4 group (T2D rats exposed to 0.010 mg/dL iAS). Again, caspase-3 activity was prompted by atorvastatin treatment. This evidence contradicts several hypotheses regarding the anti-apoptotic effect of the atorvastatin [41,42]. With respect to caspase-8, these are proteins that activate or initiate apoptosis and are synthesized within the cell as inactive pro-enzymes (procaspases). They are activated during the programmed cell death process, mainly by the extrinsic pathway [43]. We did not observe caspase-8 activity in any groups, but procaspase-8 was present in groups G1, G2, G4 and G6 with different intensity patterns (Table 3 & Fig. 1). An important characteristic of procaspase-8 activity is that groups G1 and G2 are comprised of healthy rats and groups G4 and G6 are comprised of T2D rats. In these groups, procaspase-8 was present in a higher intensity, which indicates that cells did not show apoptosis because caspase-3 was not found. The G1 group (the control) may have an apoptosis regulation mechanism because there is a antiapoptotic bcl-2 protein in this group, such that the caspase-8 would perhaps be regulated by a master anti-apoptotic regulator. The (c-FLIP) blocks procaspase-8, not allowing the release of caspase-8. Similar effects would occur with group G2 (T2D rats) presented with this disease; an apoptosis regulation mechanism may occur from a similar mechanism [44]. The G4 group showed procaspase-8 activity, but did not show caspase-8 activity. Perhaps we can interpret that the apoptosis pathway is modulated by the mechanism of inhibition of caspase-8 induction by c-FLIP. But, remembering that this group presented caspase-3 activity and low bcl-2 levels, which indicate an apoptotic process probably by the mitochondrial route and not by the caspase-8 pathway, has to do with the extrinsic activation of apoptosis. Then, 0.010 mg/dL of iAs activates caspase-3 by the mitochondrial route, inducing apoptosis of the pancreatic cells [45]. Fas is an important protein receptor of cellular death in the extrinsic pathway [46]. Fas proteins were found in the G1, G2, G4, G6, G7 and G8 groups with different blot intensities and were minor in the G1 and G2 groups. Again, this indicates an apoptosis control mechanism. Although they have Fas and procaspase-8, they do not have caspase-8, which indicates that apoptosis is not taking place. The G4 group (T2D rats exposed 0.010 mg/dL iAS) showed a greater activity compared to the G2 group (T2D rats). They did not show caspase-8 but did show positive activity for procaspase-8, indicating that it could be the beginning of an apoptotic process regulated by c-FLIP. Fas protein was also found in groups G6, G7 and G8 (T2D rats with atorvastatin). However, in these groups, caspase-8 activity was absent and caspase-3 showed positive activity. Thus, we can say that there is an apoptosis control via extrinsic processes, but it may be activated intrinsically because these groups showed a presence of caspase-3. Finally, a previous report regarding the antiapoptotic effect of atorvastatin on mouse brain tissues showed a

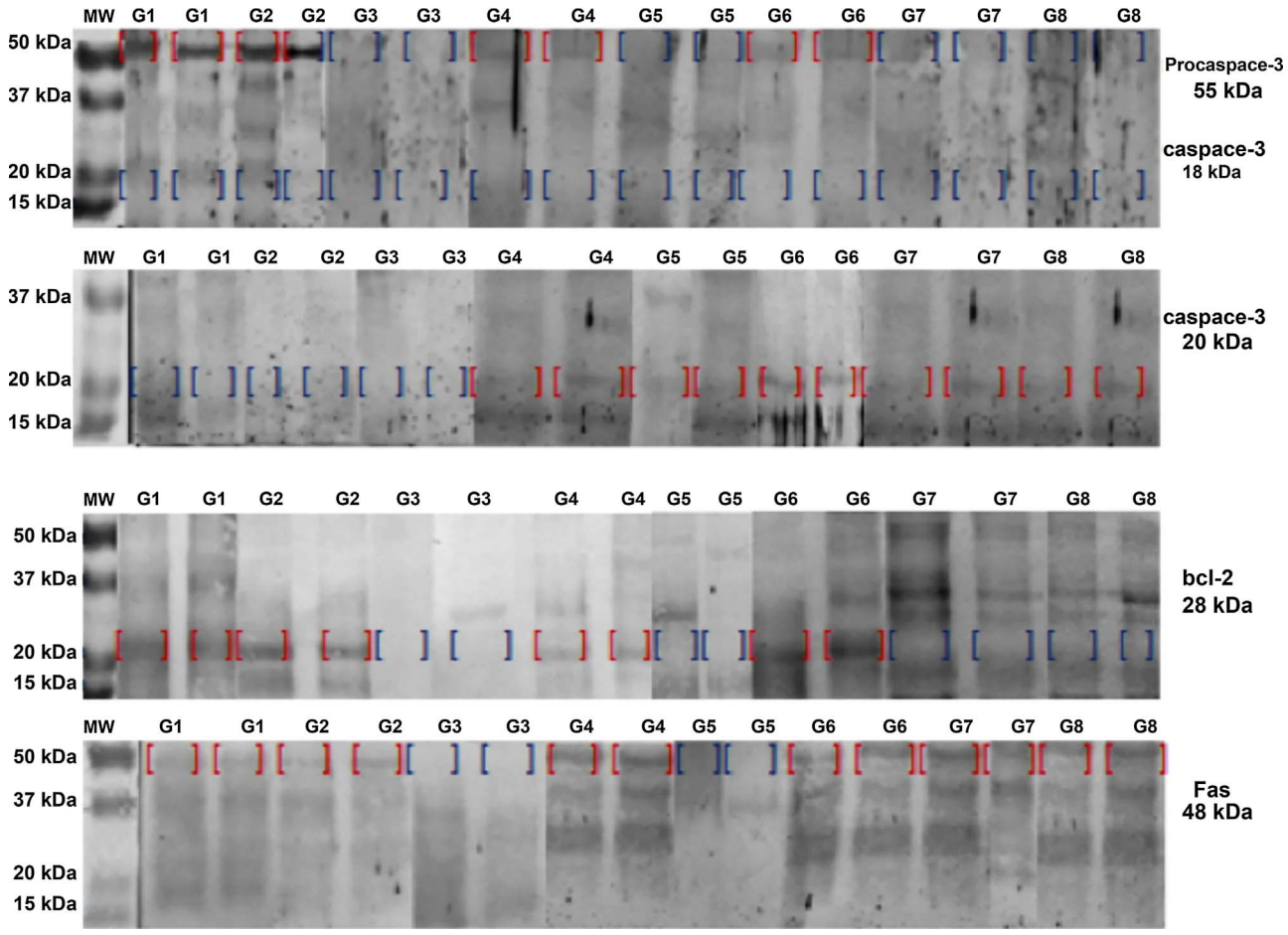

Fig. 1. Expression of procaspase-8, caspase-8, caspase-3, Bcl-2 and Fas proteins in nitrocellulose membrane.

decrease in Fas and caspase-3 expression [47]. We report that Fas is expressed in groups that were exposed to atorvastatin, and a Fas receptor and Caspase-3 were expressed in T2D groups that were exposed to atorvastatin. So, this may indicate that atorvastatin could be an initiator of the apoptosis process.

5. Conclusions

Our results suggest that iAS and atorvastatin, alone or together, are capable of activating an apoptotic process in pancreatic β-cells via caspase-3 in experimental animal models with T2D. All of these factors together cause an increase of apoptosis in pancreatic cells.

Disclosure statement

The authors report no declarations of interest.

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Involvement of *MTHFR* and *TPMT* genes in susceptibility to childhood acute lymphoblastic leukemia (ALL) in Mexicans

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Abstract

Background: Folate metabolism plays an essential role in the processes of DNA synthesis and methylation. Deviations in the folate flux resulting from single-nucleotide polymorphisms in genes encoding folate-dependent enzymes may affect the susceptibility to leukemia. This case-control study aimed to assess associations among *MTHFR* (C677T, A1298C) and *TPMT* (*2, *3A) mutations as well as to evaluate the synergistic effects of combined genotypes for both genes. Therefore, these genetic variants may lead to childhood acute lymphoblastic leukemia (ALL) susceptibility, in a Mexican population study.

Methods: DNA samples obtained from 70 children with ALL and 152 age-matched controls (range, 1–15 years) were analyzed by real-time reverse transcription polymerase chain reaction (RT-qPCR) to detect *MTHFR* C677T and A1298C and *TPMT**2 and *TPMT**3A genotypes.

Results: The frequency of the *MTHFR* A1298C CC genotype was statistically significant (odds ratio [OR], 6.48; 95% 95% confidence intervals [CI], 1.26–33.2; p=0.025). In addition, the combined 677CC+1298AC genotype exhibited a statistically significant result (OR, 0.23; 95% CI, 0.06–0.82; p=0.023). No significant results were obtained from the *MTHFR* (C677T CT, C677T TT) or *TPMT* (*2, *3A) genotypes. More importantly, no association between the synergistic effects of either gene (*MTHFR* and/or *TPMT*) and susceptibility to ALL was found.

Conclusions: The *MTHFR* A1298C CC genotype was associated with an increased risk of developing childhood ALL. However, a decreased risk to ALL with the combination of *MTHFR* 677CC+1298AC genotypes was found.

Keywords: acute lymphoblastic leukemia; *MTHFR*; *TPMT*.

Introduction

The precise mechanism of tumorigenesis is not yet resolved, but it is likely to include genetic and environmental interactions as main risk factors [1]. Kandy and Vadakedath [2] describe that folic acid and methionine play a significant role in DNA methylation and nucleotide synthesis. Therefore, folate pathway might participate in the development of carcinogenic processes. One of the main components of folate pathway is encoded by the methylene-tetrahydofolate-reductase (*MTHFR*) gene, which is located on chromosome 1p36.3 and consists of 11 exons. The gene product allows the generation of methyl donors in the synthesis of *S*-adenosyl-methionine (SAM), which is involved in DNA methylation reactions [3].

Skibola et al. [4] identified more than 20 mutations in *MTHFR*, including mutations that caused the transitions C677T (Ala22Val) and A1298C (Glu429Ala), which have been well characterized due to the severe enzymatic deficiency they cause.

An association between functional polymorphisms in the *MTHFR* gene and leukemogenesis has been demonstrated [5]; also, a folate deficiency that increased DNA damage and tumorigenicity *in vitro* has been proposed [6].

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However, findings regarding *MTHFR* polymorphisms and risk to lymphoma, cervical cancer, and acute lymphoblastic leukemia (ALL) have been reported to differ among populations [4, 7–9].

Moreover, Arenas et al. [10] suggested for the first time, that SAM (a thiopurine methyltransferase [*TPMT*] cofactor) and an *MTHFR* enzyme involved in *MTHFR* biosynthesis influence the *TPMT* activity [10]. Furthermore, it has been proposed that the binding of SAM stabilizes the enzyme's three-dimensional structure and thus affects the therapy's outcome [11]. This is due to *TPMT*, which regulates the elimination of anticancer drugs including azathioprine, 6-mercaptopurine, and 6-thioguanine [12].

The TPMT enzyme is encoded by the *TPMT* gene, which is located on chromosome 6p22.3 and consists of 10 exons. Single-nucleotide polymorphisms (SNPs) in the *TPMT* gene, including *TPMT*2* (G238C), *TPMT*3A* (G460A and A719G), *TPMT*3B* (G460A), and *TPMT*3C* (A719G) have been described [13]. The mutations G460A and A719G are usually inherited together as the *TPMT*3A* allele; thus, heterozygote or homozygote individuals for these variants might be exceptionally sensitive to the myelosupressive drug effects and the carcinogenic potential risks associated [13].

To date, no association among the *TPMT*2* and *TPMT*3C* polymorphisms and ALL susceptibility has been reported in any previous research study [14].

Therefore, to the best of our knowledge, this present study is the first report to evaluate the combined effects of *MTHFR* and *TPMT* SNPs and susceptibility to ALL in Mexicans. Consequently, this case-control study aimed to assess associations of the *MTHFR* (C677T, A1298C) and *TPMT* (*2, *3A) polymorphisms and to evaluate the synergistic effects of combined genotypes for both genes with susceptibility to childhood ALL in a Mexican population study.

Materials and methods

Patients

Pediatric patients were recruited from two hospitals in México (El Centro de Oncología Pediátrica de Baja California and El Centro Estatal de Cancerología de Durango) – these two centers are located in cities from the northern region of the country. The case group included 70 children with medical diagnosis of ALL (41 boys and 29 girls) with a mean age of 6.9 years (range, 1–15 years).

The age-matched control group included 152 randomly selected individuals (88 boys and 64 girls) with a mean age of 6.7 years. Subjects with history of malignant neoplasms were excluded. Both groups were recruited from March 2013 to July 2014. Children's parents or legal tutors were previously informed about the study's aims, and they provided written consent to participate.

This study was reviewed and approved by the Ethics and Research Committees from both hospitals, in accordance to the ethical principles of the Declaration of Helsinki [15].

MTHFR and *TPMT* genotyping

From each volunteer, 5 mL of peripheral venous blood was collected in EDTA-supplemented tubes. Genomic DNA was extracted from whole venous blood using a QIAmp DNA Blood Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). DNA integrity was confirmed by 1% agarose gel electrophoresis and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Genotyping was carried out using semiquantitative real-time polymerase chain reaction (qRT-PCR) in a StepOne Real-Time PCR system (v.2.2; Applied Biosystems, Foster City, CA, USA) under standard conditions. MGB TaqMan probes were used to identify *MTHFR* C677T (C_1202883_20), *MTHFR* A1298C (C_850486_20), *TPMT*2* (C_12091552_30), and *TPMT*3A* (C__19567_20, C_30634116_20) polymorphisms.

Statistical analysis

Analysis of the Hardy-Weinberg equilibrium (HWE) for genotype distribution was calculated using an exact test, the χ^2 test. Pearson χ^2 test was also used to estimate genotypic differences and allele frequencies between case and control groups. The odds ratios (ORs) of *MTHFR* and *TPMT* genotypes were used to test for statistically significant associations between these SNPs and ALL susceptibility. All analyses were carried out using the Statistical Analysis System software v.8.2 (SAS Institute, Cary, NC, USA). The threshold for statistically significant differences was set at $p<0.05$.

The sample size was determined based on the frequency of exposure among cases and controls with 95% confidence intervals (CI) and $\beta=0.80$ along with two controls for each case; these estimates were based on the formula by Pértega Diaz and Fernández [16].

Results

The genotype distributions of the *MTHFR (*C677T and A1298C) and *TPMT* (*2 and *3A) polymorphic loci did not deviate from HWE, except for *MTHFR* 1298 genotype in the ALL group. The genotypic frequencies for the *MTHFR* A1298C polymorphism are shown in Table 1; the frequency of the CC genotype for the A1298C polymorphism was statistically significant (OR=6.48; 95% CI, 1.26–33.2; p=0.025), suggesting an association of this mutation with ALL.

In the case of *MTHFR* C677T (also shown in Table 1), none of the genotypes showed statistically significant differences between the case and the control groups.

Table 1: Frequencies of *MTHFR* C677T and A1298C polymorphisms in the study subjects.

Genotype	ALL patients, n (%)	Controls, n (%)	OR (95% CI)	p-Value
MTHFR C677T				
CC	22 (31.4)	42 (27.6)	1.00[a]	
CT	36 (51.4)	72 (47.4)	0.95 (0.50–1.83)	0.889
TT	12 (17.2)	38 (25.0)	0.60 (0.26–1.38)	0.231
CT+TT	48 (68.6)	110 (72.4)	0.83 (0.45–1.54)	0.561
C allele	0.57	0.51		
T allele	0.42	0.49		
MTHFR A1298C				
AA	50 (71.4)	108 (71.0)	1.00[a]	
AC	14 (20.0)	42 (27.7)	0.72 (0.36–1.44)	0.351
CC	6 (8.60)	2 (1.30)	6.48 (1.26–33.2)	0.025
AC+CC	20 (28.6)	44 (29.0)	0.98 (0.53–1.84)	0.954
A allele	0.81	0.84		
C Allele	0.19	0.16		

[a]As a reference group. Allelic frequencies=(homozygote alleles×2+heterozygote alleles)/total subjects×2.

Therefore, this variant is not associated with either risk or protective effect on ALL in Mexicans.

Further analysis of the combined effect for the *MTHFR* C677T and *MTHFR* A1298C genotypes was performed (Table 2). This analysis revealed that the combined 677CC+1298AC genotypes resulted in the haplotype with protective effect on ALL (OR=0.23; 95% CI, 0.06–0.82; p=0.023, corresponding to a reduced risk of 4.3-fold with 95% CI, 1.2–16.7).

Surprisingly, our data revealed no association of the *TPMT**2 and *TPMT**3A polymorphisms with childhood ALL in this Mexican sample (Table 3).

To investigate the synergistic effect for both genes in association with leukemia, two analyses were implemented: first, the *MTHFR* C677T with the *TPMT**2 and *TPMT**3A polymorphisms; second, the *MTHFR* A1298C with the *TPMT* genotypes. Both analysis combinations showed no significant associations to ALL.

Discussion

Herein, we detected a significant association between the *MTHFR* 1298CC genotype and the risk to ALL (OR=6.48; 95% CI, 1.26–33.2; p=0.025). Also, the Hardy-Weinberg test showed a marked equilibrium in the sample of controls; however, a deviation from the HWE arose from the polymorphism *MTHFR* A1298C in the case group, suggesting a real association between its CC genotype and ALL disease [20, 21].

Moreover, this study found a protective effect of the combined genotypes of *MTHFR* 677CC+1298AC. Such findings suggest that children with the wild-type genotype for the variant 677 in combination with the heterozygous genotype for 1298 of *MTHFR* polymorphisms appear to have a decreased susceptibility to ALL (OR=0.23; 95% CI, 0.06–0.82; p=0.023), whereas the *MTHFR* 677CT, *MTHFR* 677TT, and *TPMT**2 and *TPMT**3A genotypes did not significantly influence the risk in this Mexican population. In addition, the synergistic effect for both genes (*MTHFR* and *TPMT*) showed no association to ALL susceptibility. Thus, this present study is the first report to evaluate the combined effects of *MTHFR* and *TPMT* variants and susceptibility to ALL in Mexican patients.

Concerning gene association to ALL, our results are in concordance with those of Li et al. [22], who concluded that the A1298C polymorphism (AC heterozygous genotype) represents a high risk factor to ALL in Chinese population (OR, 2.08; 95% CI, 1.13–3.84). In addition, our findings are consistent with those reported by Zanrosso et al. [23], who studied Brazilian non-White children and concluded that

Table 2: Frequencies of combined *MTHFR* C677T and A1298C genotype polymorphisms in the study subjects.

MTHFR C677T/A1298C	ALL patients, n (%)	Controls, n (%)	OR (95% CI)	p-Value
	70 (100.0)	152 (100.0)		
677 CC/1298 AA	13 (18.6)	17 (11.2)	1.00[a]	
677 CC/1298 AC	4 (5.71)	23 (15.1)	0.23 (0.06–0.82)	0.023
677 CC/1298 CC	5 (7.14)	2 (1.30)	3.27 (0.54–19.6)	0.195
677 CT/1298 AA	25 (35.7)	54 (35.6)	0.61 (0.26–1.44)	0.254
677 CT/1298 AC	10 (14.3)	18 (11.8)	0.73 (0.25–2.09)	0.553
677 CT/1298 CC	1 (1.42)	0 (0.0)[b]	3.88 (0.14–103.1)	0.416
677 TT/1298 AA	12 (17.1)	37 (24.4)	0.42 (0.16–1.12)	0.083
677 TT/1298 AC	0 (0.0)[b]	1 (0.60)	0.43 (0.01–11.4)	0.615

[a]As s reference group. [b]Where zeros cause problems with computation of the odds ratio or its standard error, 0.5 was added to all cells (a, b, c, and d) [17–19].

Table 3: Frequencies of *TPMT***2* and *TPMT***3A* (460G>A and 719A>G) polymorphisms in the study subjects.

Genotype	ALL patients, n (%)	Controls, n (%)	OR (95% CI)	p-Value
TPMT				
*1/*1	69	149	1.00[a]	
*1/*2	1	3	0.72 (0.07–7.05)	0.777
*2/*2	0[b]	0[b]	2.15 (0.04–109.5)	0.702
*1	0.99	0.99		
*2	0.01	0.01		
*1/*1	65	138	1.00[a]	
*1/*3A	5	13	0.82 (0.28–2.39)	0.711
*3A/*3A	0[b]	1	0.70 (0.02–17.5)	0.831
*1	0.96	0.95		
*3A	0.04	0.05		

[a]As a reference group. Allelic frequencies=(homozygote alleles×2+heterozygote alleles)/total subjects×2. [b]Where zeros cause problems with computation of the odds ratio or its standard error, 0.5 was added to all cells (a, b, c, and d) [17–19].

the 1298C allele (CC homozygous genotype) is also a risk factor to ALL (OR, 2.01; 95% CI, 1.01–3.99).

Concerning this study, it is evident that in Mexican population, the frequency of this polymorphism is higher than those previously reported in other populations (OR, 6.48; 95% CI, 1.26–33.2).

Conversely, other studies failed to demonstrate this aforementioned association in different study populations [9, 24, 25]. Thus, we consider several reasons for these inconsistent results: first, the *MTHFR* genetic distribution in different populations; second, plasma folate levels associated with food intake and nutritional habits; third, the possibility that ALL susceptibility may be regulated by other folate-related genes such as *TPMT*, *RFC1*, *NNMT*, and *SHMT1* [14, 26, 27].

It is noticeable that the children with the CC homozygous genotype for the *MTHFR* 1298 polymorphism have risk of leukemia, but the children who present the *MTHFR* 677 CC (wild type) and the *MTHFR* 1298 CT (heterozygous) combination may have a protective effect on leukemia (OR=0.23; 95% CI, 0.06–0.82; p=0.023).

Taking into account the striking difference, it is possible that the homozygous individuals for *MTHFR* variants would have an insufficient intracellular distribution of folate, which is necessary to maintain the three-dimensional protein structure. As a result, the production of aberrant DNA methylation and nucleotide synthesis would increase the risk to ALL development [28, 29]. Therefore, low intracellular levels of folate products of *MTHFR* genetic variants represent an important topic for future research in this population, due to reports of scarce consumption of green leafy vegetables rich in folic acid in Mexicans [30].

Accordingly, folate metabolism is involved in carcinogenesis due to its participation in DNA methylation by 5-methyl-THF [31]. *MTHFR* is responsible for the reduction reaction from 5,10-methylenetetrahydrofolate (5,10-methylene-THF) to 5-methyltetrahydrofolate (5-methyl-THF) [31], the predominant circulatory form of folate [32]. Thus, Bagley and Selhub [33] examined the effect of *MTHFR* C677T genotype on red blood cell (RBC) folate content and its relative form of distribution from groups of 677TT (homozygous) and 677CC (wild type) individuals by HPLC method. They found that the folate content in RBCs of wild-type subjects (CC) is exclusively composed of 5-methyl polyglutamates, but in the case of homozygous subjects (TT), the folate content is composed of formylated THF polyglutamates. Therefore, this genotype is associated with an incorrect distribution of folates in RBCs, and consequently, the production of 5-methyl-THF leads to variations in cellular structures of one-carbon folate products. Accordingly, a plausible explanation for our results is that *MTHFR* 677 wild-type genotype individuals could have a precise effect on nucleotide synthesis by increasing the availability of 5,10-methylene-THF necessary for normal DNA synthesis and cell division.

Additionally, the A1298C *MTHFR* polymorphism is located in the region encoding the N-terminal catalytic domain; thus, individuals with the homozygous genotype for this variant do not have the enzymatic properties distinguishable from the wild type [34].

Meanwhile, the *MTHFR* C677T variant is located in the region encoding the C-terminal SAM regulatory domain of the enzyme. It has been suggested that *MTHFR* A1298C may act through a different pathway than *MTHFR* C677T [35, 36]. Based on this hypothesis, Krajinovic et al. [37] proposed that the variant A1298C tends to accumulate 5,10-methyl-THF, whereas the C677T variant tends to accumulate 5,10-methylene-THF. The accumulation of 5,10-methylene-THF might result in 5,10-methyl-THF/5,10-methylene-THF balance. We also agree and suggest that the amount of 5,10-methyl-THF, 5,10-methylene-THF, or 5,10-methyl-THF/5,10-methylene-THF could vary depending on each genotype of independent allelic variants or combined. According to this, we found that the combined genotypes of *MTHFR* 677CC+1298AC have a protective effect on leukemia (OR=0.23; 95% CI, 0.06–0.82; p=0.023, corresponding to a reduced risk of 4.3-fold with 95% CI, 1.2–16.7). Our results confirm and extend previous findings of Krajinovic et al. [37], who reported that the combined genotypes of *MTHFR* 677TT+1298AA and 677CC+1298CC were associated with a reduced risk of developing ALL.

Thus, it is noticeable that in their results, the protective effect occurs only when the combination of genotypes integrates the homozygous/wild-type or wild-type/homozygous genotypes in each case, with the OR values (OR=0.4; 95% CI, 0.2–0.9; and OR=0.3; 95% CI, 0.1–0.6; corresponding to a 2.5- and 3.3-fold reduced risk, respectively) being lower than the those reported in this study (4.3-fold). It can be said that the combination of wild-type/heterozygous (*MTHFR* 677CC+1298AC) reported in this study confers a greater protective effect on ALL than in the case of homozygous/wild-type (*MTHFR* 677TT+1298AA) or wild-type/homozygous (*MTHFR* 677CC+1298CC) genotypes reported in the study of Krajinovic et al. [37]. There may be two reasons for this outcome: first, it is possible that the folate amount in these genotype combinations is variable but sufficient to produce a correct thymidylate synthesis, which decreases uracil disincorporation [38]; second, the genetic variability in different populations [8, 9, 24, 25].

With respect to *TPMT*, the variants *TPMT**2 and *TPMT**3A were not implicated in genetic susceptibility to ALL; these data confirm and extend previous findings of Ouerhani et al. [14].

It is noticeable that a null association for the synergistic effect of both genes studied was found; however, future research in other populations will be necessary to further support our results. More importantly, to date, there are no reports focused on associations with synergistic effects of both genes and risk to ALL in children. There is only one report where the *MTHFR* 1298AC genotype appeared to reduce ALL risk in Chinese females and males, whereas the *MTHFR* 677TT and TS2R3R/2R2R genotypes increased ALL risk in Chinese adults with low folate intake [39]. There is also another study that shows lack of association for both genes and risk to sinusoidal obstruction syndrome in ALL patients with thioguanine exposure therapy [40]. In reference to dietary and environmental factors, low folate intake may modify ALL risk [28–30, 39] and coexistence of 677TT and 1298CC alleles may elevate toxicity risk by methotrexate treatment in pediatric ALL patients [41].

In conclusion, study and comprehension of the combination of genetic and environmental factors involved in folate-dependent enzyme activity is quite promising for treatment of childhood ALL patients. Therefore, our data suggest that the *MTHFR* 1298CC genotype is associated with susceptibility to childhood ALL and, at least in Mexicans, may represent a genetic marker for childhood ALL patients. Additionally, the presence of the *MTHFR* 677CC+1298AC haplotype confers increased protection (4.3-fold) against malignancy.

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The relationship between blood lead levels and occupational exposure in a pregnant population

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Abstract

Background: Pregnant women exposed to lead are at risk of suffering reproductive damages, such as miscarriage, preeclampsia, premature delivery and low birth weight. Despite that the workplace offers the greatest potential for lead exposure, there is relatively little information about occupational exposure to lead during pregnancy. This study aims to assess the association between blood lead levels and occupational exposure in pregnant women from Durango, Mexico.

Methods: A cross-sectional study was carried out in a population of 299 pregnant women. Blood lead was measured in 31 women who worked in jobs where lead is used (exposed group) and 268 who did not work in those places (control group). Chi-square test was applied to compare exposed and control groups with regard to blood lead levels. Odds ratio (OR) and 95% confidence intervals (CI) were calculated. Multivariable regression analysis was applied to determine significant predictors of blood lead concentrations in the exposed group.

Results: Exposed women had higher blood lead levels than those in the control group (4.00 ± 4.08 μg/dL vs 2.65 ± 1.75 μg/dL, $p = 0.002$). Furthermore, women in the exposed group had 3.82 times higher probability of having blood lead levels ≥ 5 μg/dL than those in the control group. Wearing of special workwear, changing clothes after work, living near a painting store, printing office, junkyard or rubbish dump, and washing the workwear together with other clothes resulted as significant predictors of elevated blood lead levels in the exposed group.

Conclusions: Pregnant working women may be at risk of lead poisoning because of occupational and environmental exposure. The risk increases if they do not improve the use of protective equipment and their personal hygiene.

Keywords: Blood lead, Occupational exposure, Pregnant women, Risk factors

Background

Lead has been clearly shown to be a neurotoxic agent widely distributed in the environment [1]. Excessive lead exposure may occur in the workplace. Some jobs that expose people to lead include: mining, smelting, foundry work, construction, plumbing, radiator manufacturing, lead-acid battery recycling, manufacturing of rubber products, and the chemical industry. Years ago, lead was also used regularly in paint, ceramics, and pipe solder among other things. Because of its potential health problems, the amount of lead used in these products today has lessened or has been removed. However, lead is still common in many industries, including construction, mining, and manufacturing [2].

Lead can harm many of the body's organ systems. Human exposure to lead can result in a wide range of biological effects [3]. It is well known that childhood and

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pregnancy are the most sensitive population to lead exposure. A pregnant woman with an elevated blood lead concentration may expose her fetus to the toxic effect of lead. Elevated blood lead levels (BLLs) in children cause learning and behavioral deficits [4, 5]. Low-level lead exposure, including prenatal exposure, has been linked to decreased performance on IQ tests for school children [6–9]. Several studies have suggested that any level of exposure is potentially detrimental and no threshold for these effects has been identified [10, 11].

Lead concentrations have declined in the last decades due to the increase in health interventions [12]. In spite of this, lead exposure remains a risk factor for female reproductive health, even at low levels of lead in blood [13]. Once absorbed from the gastrointestinal tract or the respiratory system, lead is transported bound to erythrocytes and accumulates in bone [14]. During pregnancy, calcium demands increase. This leads to increased bone turnover, with a consequential release of lead from bone and increased blood lead levels [15, 16]. Lead can cross the placenta and expose the fetus to the harmful effects of this toxic, thus affecting the embryonic development of multiple organs and causing neurobehavioral impairments in infancy and early childhood [4, 5, 9, 17]. Therefore, pregnancy is considered a critical time for exposure to lead for the mother and the fetus [14, 18].

Over the past several decades there has been a remarkable reduction in environmental sources of lead and a decreasing trend in the prevalence of elevated blood lead levels [2]. However, some reproductive health damages at levels of lead in blood below 10 μg/dL have been reported. Therefore, in recent years, many studies have focused on the health effects at low levels of lead in blood. Low blood lead concentrations in pregnant women have been associated with miscarriage [19, 20], pregnancy hypertension, or preeclampsia [12, 21–24] premature delivery [13], premature rupture of the membranes [25], and low birth weight [26, 27]. On the other hand, it is considered that lead-related toxicity can occur at levels as low as 5 μg/dL [28]. Hence, maternal exposure to lead plays an important role in adverse pregnancy outcomes.

Despite that the workplace offers the greatest potential for lead exposure, there is relatively little information about the occupational exposure to lead during pregnancy. It is necessary to identify sources of lead exposure relevant to this population. Some of the jobs that commonly involve lead exposure are battery manufacture or repair; construction (welding or cutting lead-painted metal); radiator manufacture or repair; wire cable cutting and manufacture, and cable, battery, or scrap metal salvage, plating operations; manufacturing or using leaded paints, dyes or pigments, or lead soldering in the electronics industry, among others [29]. In Mexico, and in other developing countries, it is common to find pregnant women working in places with potential sources of lead exposure. The aim of this study was to assess the association between blood lead levels and occupational exposure in pregnant women from Durango, Mexico.

Methods

Study population

From June 2007 to May 2008 a cross-sectional study was conducted to evaluate the association between BLLs and some risk factors in pregnant women who received health attention in the State of Durango, Mexico [30]. The study population consisted of pregnant women who received medical attention in two sanitary jurisdictions pertaining to the Secretary of Health. The total estimated number of pregnant women seen in these two jurisdictions during a 1 year period was obtained from the Secretariat of Health databases, and the sample required was distributed equally in 12 municipalities. The participants were recruited from Obstetrics and Gynecology Departments of the municipal hospitals. All women who presented for prenatal care on the days that the study team visited, independent of their gestational age, were asked to participate in the study if they met the inclusion criteria. The inclusion criteria were: being pregnant, living in Durango, able to understand Spanish, and receiving health care paid for by the Secretary of Health. Each municipality was visited two or three times during the recruitment period, until the sample size was completed. Of the 337 pregnant women who presented for prenatal care on the days of the visits, 12 women were excluded because they did not live in Durango and 26 declined to participate in the study. A total of 299 women were included in the study (Aditional file 1). The interviewer's interaction with patients was standardized. All patients gave their informed written consent and answered a set of questions in a face-to-face interview. The research protocol was approved by the Ethical Committee of Durango General Hospital.

First, the group was treated as a cohort. After that, a regression with lead levels as outcome allowed to attribute the proportion of risk from occupational and non-occupational exposure. For assessment of the association between blood lead levels and occupational exposure, subjects were classified into two groups: women who worked in places where lead is used (exposed group) and women who did not work in those places (control group). Women who worked in automotive repair shops, mining laboratories, welding workshops, automotive harness factories, hairdressing salons, and road sweepers were included in the exposed group. Unemployed women and those women who had a job where lead-containing materials are not used, were included in the control group.

Blood lead measurement

Blood samples were collected using lead-free tubes containing EDTA. Samples were stored in the original tube at 4° C before being transferred to the Environmental Toxicology Laboratory, Faculty of Medicine, Juarez University of Durango State. The time between receipt and analysis varied from 1 to 3 weeks. During which time, the specimens were stored refrigerated at 4 °C. Lead concentration was determined by graphite furnace atomic absorption spectrometry. Bovine blood obtained from the National Institute of Standards and Technology (NIST) was used as standard reference material.

Statistical analysis

Data were analyzed to describe demographic characteristics, BLLs, and potential sources of lead exposure. The normality of the variables was tested using the Kolmogorov-Smirnov test. BLLs were log-transformed prior to analysis. Multivariable regression analysis was conducted to determine the proportion of risk from each occupational and non-occupational exposure. After that, the study population was divided into two groups according to occupation (occupationally exposed and non-occupationally exposed). Student *t*-test was applied for comparison of quantitative variables. Chi-square test was applied to compare exposed and control groups regarding blood lead levels (BLLs ≥ 5 μg/dL vs BLLs < 5 μg/dL). Odds ratio (OR) and 95% confidence intervals were calculated. To identify non-occupational sources of lead exposure for pregnant women we explored the following: the way in which workwear is washed (together with other clothes or alone), use of lead-glazed pottery, use of hair dyes, living near workplaces where lead is used (mining zones, battery workshops, junkyards, rubbish dumps and painting workshops), pica behavior and living with someone who works with lead, in both exposed and control groups. These activities have been documented to be lead-related. Chi-square test was also used to compare both groups regarding non-occupational sources of lead exposure. Student *t*-test was also used to compare blood lead levels according to some protection habits in the exposed group. Use of respiratory protective equipment, habit of wearing gloves, wearing of special workwear, handwashing before eating, changing clothes after work, and use of any protective equipment were analyzed as dichotomous variables. Finally, backward stepwise multivariable regression analysis was applied to determine significant predictors of blood lead concentrations in the exposed group. A set of variables selected on the basis of previous knowledge or because of associations with lead levels in bivariate analyses (at $p < 0.25$) were entered into the model. The full model was followed by stepwise backward elimination to determine whether each variable remained significant after non-significant covariates were excluded. All statistical analyses were performed using SPSS for Windows statistical package version 15.0. A p-value < 0.05 was considered statistically significant.

Results

The mean blood lead concentration in the study population was 2.79 μg/dL (SD 2.14), geometric mean 2.38 μg/dL, 95% CI (2.25 – 2.54). Among the 299 pregnant women enrolled in the study, 31 (10.4%) worked in places where lead is used, and 268 (89.6%) did not work where lead-containing materials are used (Table 1). Results of multiple linear regression on association between blood lead levels and risk factors are shown in Table 2. Living in a mine zone was associated with increased blood lead ($p = 0.044$). However, working in places where lead is used was the main factor associated with blood lead concentration. On the basis of this result, the study population was divided into two groups: exposed and non-exposed.

Table 3 summarizes the main characteristics of both groups. There were no significant differences between the groups regarding age, gestational age, number of pregnancies, body mass index (BMI), hemoglobin and monthly income per person. However, the blood lead concentration of the exposed group was significantly higher than that of the control group ($p = 0.002$).

Frequency of BLLs ≥ 5 μg/dL is depicted in Table 4. The proportion of women with BLLs ≥ 5 μg/dL in the exposed group was significantly higher compared to the control group (22.6% vs 7.1%; $p < 0.01$). In addition, women in the exposed group had 3.8 times more

Table 1 General information and blood lead levels of study population ($N = 299$)

Variables	Percent	Mean (SD)
Age (years)		24.32 (6.71)
Gestational age (weeks)		24.07 (8.68)
Pregnancies		2.0 (1.0)
Body mass index (kg/m^2)		27.23 (5.63)
Hemoglobin (g/dL)		12.55 (1.34)
Monthly income per person, USD		140.95 (144.73)
Working in places where lead is used		
Yes	10.4	
No	89.6	
Blood lead levels (μg/dL)		2.79 (2.14)
Geometric mean (95% CI)		2.38 (2.25 – 2.54)

Table 2 Results from the multiple linear regression analysis on the association between blood lead and risk factors

Risk factor	Coefficient β	95% CI	*p*
Washing the workwear together with other clothes	0.106	- 0.018 – 0.229	0.093
Use of lead glazed pottery	0.033	- 0.102 – 0.168	0.634
Dyeing hair	- 0.016	- 0.147 – 0.115	0.813
Living near workplaces where lead is used	- 0.021	- 0.197 – 0.156	0.818
Living near mining zone	0.237	0.006 – 0.468	0.044
Living near battery workshop	- 0.016	- 0.209 – 0.177	0.869
Living near junkyard	- 0.079	- 0.284 – 0.127	0.452
Living near rubbish dump	0.141	- 0.060 – 0.342	0.169
Living near straightening and painting workshop	0.023	- 0.172 – 0.218	0.819
Pica behavior	0.115	- 0.032 – 0.261	0.124
Living with someone who works with lead	0.056	- 0.071 – 0.183	0.387
Living near painting store	0.081	- 0.167 – 0.329	0.521
Living near printing office	- 0.120	- 0.441 – 0.201	0.461
Working in places where lead is used	0.306	0.103 – 0.509	0.003

R2 = 0.082

probability to have BLLs above 5 μg/dL than those in the control group.

Non-occupational sources of lead exposure for exposed and control groups are summarized in Table 5. The proportion of women who had the habit of dyeing their hair was significantly higher in exposed women when compared to the control group ($p = 0.010$) and the same was observed in the exposed group regarding living near workplaces where lead is used when compared with control women ($p = 0.043$). However, there were no significant differences in other variables between the compared groups.

To evaluate the influence of some work conditions on blood lead levels in the exposed group, some protection habits were explored (Table 6). Blood lead levels were significantly higher in women who did not wear special workwear ($p = 0.028$) and in those who did not have the habit of changing clothes after work ($p = 0.025$).

Table 7 displays potential sources of blood lead in the exposed group. After multivariable analysis, seven variables were retained in the final model: wearing of special workwear, changing clothes after work, living near a painting store, living near a printing office, living near a junkyard, living near a rubbish dump and washing the workwear together with other clothes. These variables accounted for 86.5% of the total variance. The model was adjusted by age, educational level and gestational age.

Discussion

In this cross-sectional study, we examined the association of blood lead levels with occupational exposure in pregnant women. The blood lead levels in our

Table 3 General information and blood lead levels of the exposed subjects and control group[a]

Variable	Exposed group ($n = 31$)	Control group ($n = 268$)	*p* value[*]
Age (years)	26.03 (6.17)	24.13 (6.76)	0.135
Gestational age (weeks)	22.71 (8.06)	24.22 (8.75)	0.358
Number of pregnancies	2.55 (1.38)	2.23 (1.47)	0.253
Body mass index (kg/m^2)	28.81 (4.79)	27.04 (5.70)	0.098
Hemoglobin (g/dL)	12.97 (1.11)	12.50 (1.36)	0.065
Monthly income per person, USD	165.62 (130.59)	138.00 (146.28)	0.316
Blood lead levels (μg/dL)	4.00 (4.08)	2.65 (1.75)	0.002[**]

[a]Values shown as mean (standard deviation)
[*] *p* value was calculated from Student *t*-test
[**] *p* value from Log BLL

Table 4 Frequencies of BLL ≥ 5 μg/dL in the study population

Subjects	BLLs ≥ 5 μg/dL n (%)	BLLs <5 μg/dL n (%)
Exposed group ($n = 31$)	7 (22.6)	24 (77.4)
Control group ($n = 268$)	19 (7.1)	249 (92.9)
Total ($n = 299$)	26 (8.7)	273 (91.3)

$X^2 = 6.56$; $p = 0.010$; OR = 3.822; 95%; IC (1.460 – 10.008)

study population (2.79 ± 2.14 μg/dL) did not exceed the accepted threshold of 10 μg/dL. They are even below the 5 μg/dL recommended by the CDC [31]. Furthermore, the mean blood lead level in our test subjects is lower compared to values reported in some populations of pregnant women. A study by Taylor et al. [14] reported mean BLL of 3.67 ± 1.47 μg/dL in a cohort of pregnant women in The United Kingdom. In China, the lead concentrations during the three pregnancy trimesters and postpartum were 5.95 ± 2.27 μg/dL, 5.51 ± 1.93 μg/dL, 5.57 ± 1.85 μg/dL, and 6.88 ± 1.90 μg/dl; respectively [32]. In addition, Gerhardsson and Lundh [33] reported median blood lead of 11.0 μg/L (range 4.2–79 μg/L) in pregnant females residing in Sweden; and Alvarez et al. [34] found a blood lead average of 11.63 ± 4.64 μg/dL in pregnant women living in the island of Tenerife, Spain. However, some researchers have reported lower blood lead concentrations in pregnant women. Mean blood lead levels of 2.551 ± 2.592 μg/dL were found in pregnant women from Saudi Arabia [35]. In a socioeconomically disadvantaged population of New York, a geometric mean of 1.58 μg/dL was reported by Schell et al. [15]. Moreover, Bakhireva et al. [36] found mean blood lead of 1.06 ± 1.55 μg/dL in a cross-sectional study designed to ascertain risk factors of lead exposure among pregnant women in New Mexico, United States.

In Mexico, the Secretary of Health is the health care institution which attends the smallest workforces. Nevertheless, we found 31 women working in places where lead is used and who represent 10.4% of the recruited subjects. In spite of this, lead in the workplace results a significant determinant of blood lead levels. Therefore, similar results may be expected in other pregnant populations with low income and low level of employment.

Our exposed group was made up of women who worked in automotive repair shops, mining laboratories, welding workshops, automotive harness factories, hairdressing salons, and as road sweepers, regardless of intensity and exposure time. At any rate, we found significantly higher blood lead concentrations in exposed women than in the control group (4.24 ± 4.60 μg/dL vs. 2.66 ± 1.73 μg/dL). Our findings are consistent with a study by Popovic et al. [37], who found mean blood lead of 2.73 ± 2.39 μg/dL in women formerly working in a smelter, and 1.25 ± 2.10 μg/dL in women with no known occupational exposure to lead.

In the present study, no difference was observed in hemoglobin level between exposed women and the control group. This is expected considering the low BLLs obtained for this population. According to previous studies, lead anemia appears at BLLs higher than 40 μg/dL [3, 38]. On the other hand, the US Environmental Protection Agency (EPA) suggests a threshold BLL of 20 – 40 μg/dL for risk of anemia [39]. However, blood lead concentrations in our compared groups are much lower.

Table 5 Comparison of non-occupational sources of lead exposure between exposed and control groups[a]

Potential source of lead exposure	Exposed group ($n = 31$)	Control group ($n = 268$)	p value*
Washing the workwear together with other clothes	12 (38.7)	123 (45.9)	0.447
Use of lead glazed pottery	10 (32.3)	81 (30.2)	0.816
Dyeing hair	27 (87.1)	172 (64.2)	0.010
Living near workplaces where lead is used	22 (71.0)	139 (51.9)	0.043
Living near mining zone	4 (12.9)	25 (9.3)	0.752
Living near battery workshop	7 (22.6)	43 (16.0)	0.356
Living near junkyard	4 (12.9)	30 (11.2)	0.777
Living near rubbish dump	3 (9.7)	39 (14.6)	0.641
Living near straightening and painting workshop	7 (22.6)	45 (16.8)	0.421
Pica behavior	10 (32.3)	62 (23.1)	0.261
Living with someone who works with lead	16 (51.6)	101 (37.7)	0.133

[a]Values shown as frequency (percentage)
*p value from Chi-square test

Table 6 Comparison of blood lead levels regarding protection habits in exposed women

Protection habits	Blood lead levels, μg/dL [a]		*p* value*
	No	Yes	
Use of respiratory protective equipment	27 (4.03 ± 4.23)	4 (3.75 ± 3.43)	0.901
Wearing gloves habit	19 (4.32 ± 4.96)	12 (3.48 ± 2.18)	0.521
Wearing of special workwear	20 (4.92 ± 4.85)	11 (2.31 ± 0.68)	0.028
Hand washing before eating	11 (3.55 ± 1.48)	20 (4.24 ± 5.00)	0.571
Changing clothes after work	24 (4.51 ± 4.52)	7 (2.24 ± 0.60)	0.025
Use of any protective equipment	9 (5.64 ± 7.03)	22 (3.32 ± 1.83)	0.356

[a] Values shown as frequency (mean ± standard deviation)
* *p* value from Student *t*-test

Recent findings concerning lead-related adverse reproductive outcomes suggested that pregnant women should avoid lead exposure that would result in blood lead concentrations higher than 5 μg/dL [3]. Among the 299 women included in our study, 26 (8.7%) had BLLs ≥ 5 μg/dL. In a cohort of 4, 285 pregnant women, Taylor et al. [14] reported 14.4% of women with BLLs of 5 μg/dL or higher; cigarette smoking, alcohol, and coffee drinking were found to be predictors of BLLs. However, in our study the frequencies of smoking, alcohol and coffee drinking among the women were very low; therefore, these variables were not included in the analysis. Regarding occupation, the 2005 – 2007 Adult Lead Epidemiology and Surveillance (ALES) by the United States of America Centers for Disease Control and Prevention reported that 32% of women of childbearing age with BLL ≥ 5 μg/dL were occupationally exposed to lead [38]. Zhu et al. [40] evaluated reasons for testing and potential sources of exposure among women, and reported that 29.2% of women with blood lead of 5–14.9 μg/dL had a job with potential lead exposure.

Our results indicated that exposed women were more than 3.8 times likely to have BLLs ≥ 5 μg/dL than non-exposed women. This finding suggests that occupation represents an important factor for elevated blood lead concentrations in our studied population. According to a study by Kosnett et al. [3], it is recommendable for pregnant women to avoid lead exposure that would result in blood lead levels above 5 μg/dL, due to the raised concerns regarding the toxicity of this blood lead concentration. Several studies have associated blood lead levels above 5 μg/dL with miscarriage [19, 20], pregnancy hypertension [12, 21–24, 41], premature delivery [13], premature rupture of the membranes [25], and low birth weight [26, 27]. According to CDC recommendations [28], pregnant women with a current or past BLL ≥5 μg/dL should be assessed for the adequacy of their diet and provided with prenatal vitamins, calcium and iron supplements.

We found a higher proportion of women living near workplaces where lead is used among exposed women compared with the control group. There was also a significant association between the BLLs and the habit of dyeing the hair. Some hair dyes may contain lead and other harmful substances. Our results agree with Marzulli [42] who reported a significant correlation between blood lead and hair lead in people who used lead contained hair dyes. Use of these products by a pregnant woman may harm the health of her unborn child. None of the cited investigations, carried out in an occupational cohort, analyzed non-occupational exposure. However, our findings suggest that the contribution of non-occupational activities must be explored for determining total lead exposure and subsequent health effects.

Table 7 Regression analysis for predictors of BLLs in exposed group (*N* = 31)

Variable	Coefficient β	95% CI	*P**
Wearing of special workwear	- 0.608	- 1.115 – -0.102	0.021
Changing clothes after work	- 0.637	- 1.261 – - 0.013	0.046
Living near painting store	3.937	1.174 – 6.699	0.008
Living near printing office	7.418	.963 – 10.873	0.001
Living near junkyard	3.661	0.691 – 6.632	0.019
Living near rubbish dump	3.469	0.036 – 6.901	0.048
Washing the workwear together with other clothes	2.372	0.267 – 4.477	0.029

$R^2 = 0.865$
* Adjusted by age, educational level and gestational age

Occupational lead exposure can occur because of the use of lead material and products. For that reason, employers should provide their employees with adequate working conditions and protection information regarding hazards at their worksites. Exposed workers should use protective equipment and practice personal hygiene, such as showering and changing into clean clothes at the end of the shift [43]. In this study, working women who did not change their clothes after work showed significantly higher blood lead concentration in comparison with those women who had this habit. There was also statistical association of BLLs related to the use of special workwear. It is well known that appropriate workwear can greatly reduce exposure to hazardous substances [44]. In addition, clothing contaminated with lead can be an important route of exposure for pregnant women.

Despite the scientific data and practical considerations regarding the prevention of lead exposure during pregnancy, routine blood lead testing for pregnant women is not established in many countries. Nevertheless, it is the main way to make sure that women have not been affected by lead. Furthermore, some researchers have demonstrated that lead exposure during pregnancy affects children's physical neonatal development, and available evidence suggests there are no BLLs without risk of health effects [41].

Relatively little is known about the current prevalence, risk factors, and sources of lead poisoning among pregnant women [45]. Our study identified some risk factors associated with blood lead in occupationally exposed women. Despite the growing evidence that relatively low levels of environmental lead exposure may be associated with adverse pregnancy outcomes, there is no specific regulation in existence regarding occupational lead exposure during pregnancy in Mexico. Therefore, it is necessary to improve engineering controls and personal hygiene to reduce the risk of lead exposure during pregnancy. Much work needs to be done to reduce environmental lead exposure. Furthermore, exposed women should undergo blood lead testing to prevent lead poisoning.

We have recognized that our study has several limitations. First, the cross-sectional design did not allow an evaluation of the length and the extent of the exposure. Consequently, all the exposed women were included in a single group, regardless of the time spent in the working place. Longitudinal studies are needed to evaluate the changes in blood lead levels during the exposure time. Second, in our study calcium supplementation, dietary iron intake and indicators of iron status were not measured. It has been documented that low calcium intake may contribute to lead mobilization from the maternal skeleton during pregnancy [46] and that calcium supplementation reduces bone resorption [47] and minimizes release of lead from bone stores with subsequent fetal lead exposure [48, 49]. On the other hand, an inverse relationship between body stores of iron and lead retention has also been observed [50, 51]. Nevertheless, to our knowledge, it is the first study on this topic conducted in occupationally exposed pregnant women in Mexico. Therefore, the results of the present research can be used for comparison with future investigations regarding occupational exposure to lead during pregnancy.

Conclusions

Our results constitute evidence that pregnant women who work in some places where lead products are used may be at risk for presenting higher blood lead levels if they do not use protective equipment and do not practice adequate personal hygiene. The risk increases if women live near some places that are considered sources of lead exposure such as a painting store, a printing office, a junkyard, or a rubbish dump. Additional studies using larger sample sizes and multiple prospective measurements are needed to verify our findings.

Additional file

Additional file 1: Database: Blood lead levels in pregnant women from Durango, Mexico. (XLS 733 kb)

Abbreviations

ALES: Adult lead epidemiology surveillance; BLLs: Blood lead levels; BMI: Body mass index; CDC: Centers for disease control and prevention; CI: Confidence interval; EDTA: Ethylenediaminetetraacetic acid; NIST: National Institute for Standard Technology; OR: Odds ratio; SD: Standard deviation

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Availability of data and material

All data analyzed during this study are included in this published article, in the Additional file 1: DatabasePb.xls.

Authors' contributions

OLLLL designed the study, participated in the elaboration of the questionnaire, prepared the background, results and discussion sections, as well as part of the methods sections, JMSP participated in the elaboration of the questionnaire, he was the field supervisor and contributed to the discussion of results, SEM collaborated in the statistical analysis and interpretation of results, EER carried out part of the literature review, participated in the process of data collection and contributed to the discussion and interpretation of results, FXCJ contributed to the discussion and interpretation of results and prepared part of the results and discussion sections, ASC participated in the elaboration of the questionnaire, in the

process of data collection and critically reviewed the manuscript, AMLQ participated in the design of the questionnaire, carried out part of the literature review and collaborated to the interpretations of results, FVA collaborated in the statistical analysis and interpretation of results, EMMH contributed to the discussion of the findings, she also contributed in drafting and writing of the manuscript, GGV supervised the procedures for blood lead measurements and contributed to the discussion of the findings. JDS contributed with blood lead measurements, interpreting the results, and providing critical comments. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
This study was approved by the Ethical Committee of Durango General Hospital. All participants gave their informed written consent before being enrolled.

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Association between Blood Lead Levels and Delta-Aminolevulinic Acid Dehydratase in Pregnant Women

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Abstract: Blood lead levels (BLLs) and delta-aminolevulinic acid dehydratase (ALAD) activity are considered biomarkers of lead exposure and lead toxicity, respectively. The present study was designed to investigate the association between BLLs and ALAD activity in pregnant women from Durango, Mexico. A total of 633 pregnant women aged 13–43 years participated in this study. Blood lead was measured by a graphite furnace atomic absorption spectrometer. ALAD activity was measured spectrophotometrically. Mean blood lead was 2.09 ± 2.34 μg/dL; and 26 women (4.1%) crossed the Centers for Disease Control (CDC) recommended level of 5 μg/dL. ALAD activity was significantly lower in women with levels of lead ≥5 μg/dL compared to those with BLLs < 5 μg/dL ($p = 0.002$). To reduce the influence of extreme values on the statistical analysis, BLLs were analyzed by quartiles. A significant negative correlation between blood lead and ALAD activity was observed in the fourth quartile of BLLs ($r = -0.113$; $p < 0.01$). Among women with blood lead concentrations ≥2.2 μg/dL ALAD activity was negatively correlated with BLLs ($r = -0.413$; $p < 0.01$). Multiple linear regression demonstrated that inhibition of ALAD in pregnant women may occur at levels of lead in blood above 2.2 μg/dL.

Keywords: blood lead levels; delta-aminolevulinic acid dehydratase (ALAD) activity; pregnant women; lead exposure; lead toxicity

1. Introduction

Lead is known to represent a significant environmental hazard to pregnant women and their offspring. Exposure to high environmental levels of lead during pregnancy has been associated with some adverse outcomes [1]. However, recent findings indicate that lead may be toxic at levels previously considered to have no adverse effects. Research suggests that lead exposure at both low

and high concentrations adversely affects hematopoietic, vascular, nervous, renal and reproductive systems [2]. During pregnancy, adverse reproductive outcomes may occur at levels of lead in blood below 10 μg/dL. Infertility [3], spontaneous abortion [4], preeclampsia [5–7] and preterm delivery [8] have all been associated with lead exposure at levels previously considered safe.

Blood lead concentrations above 2.5 μg/dL have been associated with an increased risk of infertility [3]. A significant association between blood lead concentrations and hypertension during pregnancy has been documented [5,7]. Significantly higher blood lead levels have been reported in women with pregnancy-induced hypertension compared to normotensive patients, and significant correlations between blood lead levels and systolic and diastolic blood pressures have been found [7]. Moreover, higher levels of lead in umbilical cord blood have been found in preeclampsia cases compared to women without this condition [5].

Elevated lead levels have been also associated with abortion and duration of pregnancy [4,8]. In a prospective study in Mexico city a statistically significant relationship between low-to-moderate maternal lead levels and the risk of spontaneous abortion was demonstrated [4]. Furthermore, researchers have found significantly higher blood lead levels during the first trimester of pregnancy in mothers who delivered preterm babies when compared with those whohadfull-term pregnancies [8].

Several biological techniques and biomarkers are useful for risk assessment of lead in the field of environmental health. Blood lead is the most widely used biomarker of lead exposure. This indicator represents a measure of soft tissue lead, body burden and absorbed doses of lead, whereas the critical effects of lead in bone marrow can be used as biomarker of effect. The effects of lead in bone marrow arise mainly from lead interaction with some enzymatic processes involved in heme synthesis [9].

The main biomarkers of effect are the inhibition of delta-aminolevulinic acid dehydratase (ALAD) and the variation in some metabolite concentrations, such as zinc protoporphyrin (ZP) in blood, delta-aminolevulinic acid in urine (ALA-U), delta-aminolevulinic acid in blood (ALA-B), delta-aminolevulinic acid in plasma (ALA-P) and coproporphyrin in urine (CP). However, not all mentioned indicators equally reflect dose and internal dose/effect relationship [2].

Lead toxicity may be explained by its interference with different enzymes. Lead inactivates these enzymes by binding to the SH-groups of proteins or by displacing some essential metal ions. Lead is known to inhibit three enzymes involved in the heme pathway: delta-aminolevulinic acid dehydratase, ferrochelatase, and coproporphyrinogen oxidase, but the major effectsareon ALAD activity. The δ-aminolevulinic acid dehydratase is the second enzyme of the heme pathway. This enzyme catalyzes the condensation of two molecules of δ-aminolevulinic acid (ALA) to form the monopyrrole porphobilinogen (PBG) [10]. In subsequent steeps, PBG is assembled into tetrapyrrole molecules, which constitute the prosthetic groups of hemoglobin [11]. Lead inhibition of ALAD activity results in accumulation of δ-aminolevulinic acid. ALA has been associated with oxidative damage by causing formation of reactive oxygen species (ROS), such as superoxide, hydroxyl radical, and hydrogen peroxide [12–14].

Negative correlations between blood lead concentration and ALAD activity have been reported, even at low levels of lead in blood [9,15,16]. On the other hand, positive correlations have been found between ALAD activity and malondialdehyde (MDA) levels [16]. Thus, ALAD activity is thought to be a sensitive indicator of early effect of lead as well as a biomarker of oxidative stress in the lead-exposed hematological system [17]. Blood lead has been considered a reliable indicator for the evaluation of lead exposure, whereas inhibition of ALAD activity has been considered one of the primary detectable parameters of lead poisoning [2].

Activity of ALAD is easily assayable in samples of peripheral blood. This enzyme has a high sensitivity to divalent lead ions, so it can be used as an indirect biomarker to estimate exposure to lead in humans [18]. ALAD activity test is considered appropriate for screening purposes, due to the progressive inactivation of this enzyme by lead over a range corresponding to subclinical intoxication [19]. In addition, ALAD activity is more sensitive than ALA in urine to evaluate the amount of circulating lead [9,20].

Previous epidemiological studies on the association between blood lead levels (BLLs) and ALAD activity showed divergent views. Studies reporting high levels of lead in blood revealed significant negative correlations between blood lead concentrations and ALAD activity [12,21,22]. However, some authors have demonstrated that ALAD inhibition occurs at levels of lead in blood around 5 μg/dL [15,16,23]. Most studies regarding the association between BLLs and ALAD activity have been conducted in occupationally exposed people and in children. Nevertheless, no significant variation of enzymatic ALAD activity has been reported in children at mean blood lead of 2.58 ± 0.30 μg/dL [13].

In a previous study, conducted by our research group, blood lead levels and some risk factors for lead exposure in pregnant women were determined, but ALAD activity was not evaluated [24]. The present cross-sectional study was designed to investigate the association between BLLs and ALAD activity in pregnant women from Durango, Mexico.

2. Materials and Methods

2.1. Subjects

This cross-sectional study was carried out between January 2014 and June 2016. The study subjects consisted of 633 clinically healthy pregnant women who received prenatal health care by the Secretariat of Health, State of Durango, Mexico. All pregnant women presented for prenatal care in health centers were asked to participate in the study. Those who accepted gave their written informed consent before being enrolled. Patients with renal failure, infectious disease or multifetal pregnancy were excluded. Participants were informed of the aims of the investigation and received information on ways to minimize their lead exposure. Each subject answered a questionnaire that contained sociodemographic data and information on reproductive history and sources of lead exposure. The study was conducted in accordance with the Declaration of Helsinki, and the research protocol was approved by the Ethical Committee of Durango General Hospital (approval number: 366/013).

2.2. Sample Collection

For determination of ALAD activity, a venous blood sample was drawn for each patient and collected in vacutainer tubes using sodium heparin as an anticoagulant. A second sample was collected in lead-free vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA), and separated in two portions; one for hematological analysis, and the remaining aliquot for lead level determination. Blood samples were collected before fasting. After collection, blood samples were transported in ice boxes to the Clinical Analysis Laboratory, Scientific Research Institute, Juarez University of the State of Durango. Samples were stored and transported in a lead-free environment to avoid any contamination, handled by trained personnel and kept in reserve at 4 °C.

2.3. Measurement of ALAD Activity

Enzyme activitywas assayed spectrophotometrically by the standardized European method [25]. The enzyme was incubated with excess δ-aminolevulinic acid at 37 °C. The porphobilinogen which was formed in 1 h was mixed with modified Ehrlich reagent. The color developed was measured spectrophotometrically at 555 nm against a blank. Results were expressed as δ-aminolevulinic acid, μmol/min per liter erythrocytes (U/L). The activity was determined no later than 10 h after the sample collection.

2.4. Hematological Analysis

Hematological parameters were determined using an automated hematology analyzer (Abbott CELL-DIN 1400), at the Clinical Analysis Laboratory, Scientific Research Institute, Juarez Universityof the State of Durango. Red blood cells count (RBC), hemoglobin (Hb), hematocrit, meancorpuscular volume (MCV), mean corpuscular hemoglobin, and meancorpuscular hemoglobin concentration were determined. The hematocrit value was used for the calculation of the enzyme

activity. Only hemoglobin value was presented in the results because of the possible relationship between hemoglobin and blood lead levels.

2.5. Determination of Lead in Blood

Blood samples were transferred to the Laboratory of Environmental Toxicology, Faculty of Medicine, Juarez University of the State of Durango, Gomez Palacio Campus. This laboratory participates in the Wisconsin State Laboratory Program of Hygiene proficiency testing (WSLPHT). Blood lead was measured using a graphite furnace atomic absorption spectrometer Perkin-Elmer AAnalyst 800 with Zeeman-effect background correction. Duplicates of blood samples were analyzed according to Miller et al. [26]. Lead in bovine blood from the National Institute of Standard and Technology (NIST) was used as standard reference material. Each sample duplicate was analyzed twice and those with variation coefficient above 5% were reanalyzed.

2.6. Statistical Analysis

The sociodemographic and reproductive characteristics were shown as mean ± standard deviation. The study population was divided into two groups: those with BLLs < 5 μg/dL and those with BLLs ≥ 5 μg/dL, and Student's *t*-test was used to estimate differences between groups. To reduce the influence of extreme values on the statistical analysis, blood lead levels were analyzed by quartiles. One-way ANOVA was applied to compare the means between quartiles and the post-hoc comparisons were done using Tukey's test. Pearson correlation analysis was carried out to evaluate the relationship of blood lead concentration with hemoglobin and ALAD activity in all groups. Multiple linear regression was performed to evaluate the association of ALAD activity with BLLs. Statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) software for Windows, version 15.0. A value of $p < 0.05$ was considered statistically significant.

3. Results

Table 1 summarizes the main characteristics, blood lead levels, and ALAD activity of women enrolled in this study. The mean age, education, gestational age, body mass index and hemoglobin of the studied population were 22.85 years, 10.04 years, 13.44 weeks, 25.61 kg/m^2 and 13.00 g/dL, respectively. The mean income per capita accounted 99.55 United States Dollars (USD) per month (1 USD = 17.0 Mexican pesos). The mean level of blood lead was 2.09 ± 2.34 μg/dL; and the mean ALAD activity was 57.59 ± 21.12 U/L.

Table 1. Main characteristics of the studied subjects (n = 633). ALAD: delta-aminolevulinic acid dehydratase.

Variables	Mean ± SD *	Range
Age (years)	22.85 ± 6.35	13–43
Education (years)	10.04 ± 2.67	0.0–21.0
Gestational age (weeks)	13.44 ± 4.86	3.0–28.0
Body mass index (kg/m^2)	25.61 ± 5.25	16.0–54.4
Income per capita (USD ** per month)	99.55 ± 89.68	4.41–970.59
Hemoglobin, g/dL	13.00 ± 1.27	8.8–23.1
Blood lead levels, μg/dL	2.09 ± 2.34	0.48–26.85
ALAD activity, U/L	57.59 ± 21.12	3.28–138.81

Note: * SD = standard deviation; ** USD = United States Dollars.

Table 2 shows some characteristics for women with lead levels <5 μg/dL, and for women with lead levels ≥5 μg/dL. No significant differences between the groups were observed in age, education, gestational age, body mass index, monthly income per person and hemoglobin. However, ALAD activity was significantly lower in women with lead levels ≥5 μg/dL ($p = 0.002$).

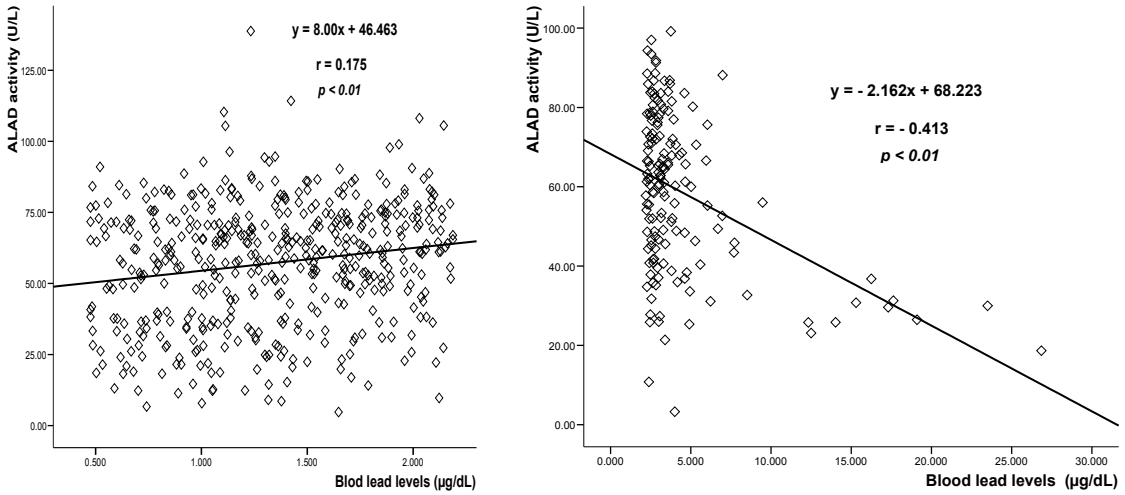
Table 3 shows sociodemographic variables, hemoglobin and ALAD activity by quartiles of blood lead. A significant variation of ALAD activity was observed ($p < 0.001$). According to the Tukey test, women in the first quartile had the lowest ALAD activity. On the other hand, enzyme activity decreased between the third and the fourth quartiles. On the basis of these results, Pearson correlation was performed to determine the relation of blood lead concentration with hemoglobin and ALAD activity by quartiles of BLLs (Table 4). The correlation of BLLs with hemoglobin was not statistically significant. However, significant negative correlation between BLLs and ALAD activity was observed in the fourth quartile (r = −0.413; $p < 0.01$).

Table 2. Main characteristics of women with blood lead levels <5 μg/dL and ≥5 μg/dL. BLL: blood lead levels.

Variables	BLLs < 5 μg/dL (n = 607)	BLLs ≥ 5 μg/dL (n = 26)	*p* *
	Mean ± SD	Mean ± SD	
Age (years)	22.87 ± 6.36	22.42 ± 6.13	0.728
Education (years)	10.06 ± 2.68	9.58 ± 2.52	0.372
Gestational age (weeks)	13.46 ± 4.85	12.95 ± 5.06	0.612
Body mass index (kg/m^2)	25.53 ± 5.20	27.36 ± 5.92	0.082
Income per capita (USD per month)	99.76 ± 78.70	94.32 ± 69.27	0.776
Hemoglobin, g/dL	13.00 ± 1.28	13.00 ± 1.04	0.974
ALAD activity, U/L	58.13 ± 21.05	45.10 ± 19.22	0.002

Note: * *p*-value was calculated from Student's *t*-test.

Taking into account the lower limit of blood lead for the third quartile, linear regression analysis was performed to determine the strength of the relationship between BLLs and ALAD activity in women with blood lead concentrations lower 2.2 μg/dL, and in those with BLLs ≥ 2.2 μg/dL (Figure 1). No significant association was observed between ALAD activity and BLLs for women with BLLs < 2.2 μg/dL. However, the results demonstrated a significant negative correlation (r = −0.413; $p < 0.01$) for women with BLLs ≥ 2.2 μg/dL.



(**A**) (**B**)

Figure 1. Linear regression between blood lead levels and δ-ALAD activity for women with BLLs < 2.2 μg/dL (**A**); and for thus with BLLs ≥ 2.2 μg/dL (**B**). The linear equation, correlation coefficient and *p* value are shown in the plot.

Table 3. Change in demographic characteristics, hemoglobin and ALAD activity by quartiles of blood lead levels.

Variables	First Quartile	Second Quartile	Third Quartile	Fourth Quartile	*p* *
n	160	158	158	157	
BLLs (μg/dL)	<1.09	1.09–1.61	1.62–2.19	>2.19	
Age, years	22.50 ± 6.84	23.60 ± 6.13	23.10 ± 6.08	23.20 ± 6.36	0.696
Education (years)	10.10 ± 2.70	10.18 ± 2.73	9.81 ± 2.60	10.05 ± 2.72	0.637
Gestational age (weeks)	13.69 ± 4.98	13.47 ± 4.73	13.71 ± 4.94	12.86 ± 4.79	0.375
Body mass index (kg/m^2)	24.90 ± 5.31	25.92 ± 5.39	26.01 ± 5.27	25.58 ± 4.98	0.254
Income per capita (USD per month)	98.41 ± 76.47	96.10 ± 100.04	95.99 ± 71.45	108.07 ± 106.64	0.614
Hemoglobin (g/dL)	12.88 ± 1.13	12.93 ± 1.20	12.95 ± 1.04	13.23 ± 1.64	0.070
ALAD activity, U/L	51.51 ± 21.82	59.10 ± 22.18	61.02 ± 19.10	58.82 ± 20.14	0.000

Note: * *p*-value was calculated from one-way ANOVA.

Table 4. Pearson correlations of blood lead levels with hemoglobin and ALAD activity by quartiles of blood lead levels.

Quartile of BLLs	Hemoglobin	ALAD Activity
First	0.027	−0.013
Second	−0.042	−0.043
Third	0.076	0.116
Fourth	−0.087	−0.413 **
All subjects	0.017	−0.113 **

Note: ** = Statistically significant correlation ($p < 0.01$).

To deepen the exploration of the relationship between blood lead concentration and ALAD activity in women with BLLs ≥ 2.2 μg/dL, multiple linear regression was applied (Table 5). Blood lead levels were inversely associated with ALAD activity ($p < 0.001$). However, no significant associations were found for age, educational level, gestational age, body mass index and hemoglobin. The model represents 21.9% of the predictive capability.

Table 5. Multiple linear regression model for ALAD activity in women with BLLs ≥ 2.2 μg/dL (n = 142).

Variable	Coefficient β	Standard Error	*p*-Value
Age, years	0.239	0.261	0.361
Educational level, years	0.689	0.578	0.235
Gestational age, weeks	0.202	0.339	0.553
Body mass index (kg/m^2)	−0.443	0.338	0.192
Hemoglobin (g/dL)	1.841	0.958	0.057
Blood lead levels (μg/dL)	−1.961	0.404	<0.001

Note: $R^2 = 0.219$.

4. Discussion

The mean blood lead concentration of 2.09 ± 2.34 μg/dL reported here is lower than those observed in other studies carried out in Mexican population. In Mexico City, Borja-Aburto found blood lead concentrations of 12.03 μg/dL in pregnant women who suffered spontaneous abortion and 10.09 μg/dL in a control group [4]. Another study of blood lead levels in pregnant women from Mexico City reported a mean blood lead concentration of 6.24 g/dL [27]. In a previous study carried out by our research group in pregnant women from Durango, Mexico, a mean blood lead level of 2.79 ± 2.14 μg/dL was observed, and 26 women (8.7%) had BLLs above the CDC recommended level of 5 μg/dL [24]. In the present research, also 26 women had levels of lead in blood above 5 μg/dL, but they represent 4.1% of the studied population.

Some authors have suggested that lead intoxication is characterized by high blood lead concentration and low ALAD activity [27,28]. For that reason, some researchers have recommended use of ALAD inhibition as an indicator of lead intoxication [12,21,29]. In our study, ALAD activity was significantly lower in women with BLLs $\geq$ 5 μg/dL compared with those with BLLs below 5 μg/dL. This finding is in an agreement with earlier published data. Similar results were observed in urban male adolescents from Lucknow, India [12], in children with neurological diseases from India [16], in lead workers from Taiwan [29], and in children from Southern Brazil [22].

Chiu et al. reported an inverse association between blood lead and ALAD activity when they compared lead workers from Taiwan with a control group (blood lead levels 19.5 $\pm$ 14.7 μg/dL and 2.9 $\pm$ 1.9 μg/dL, respectively) [29]. They concluded that the possible threshold value of blood lead for ALAD activity is around 10 μg/dL, and thus, ALAD activity may be usedas a biomarker for evaluation of lead toxicity in humans. Similar results were reported by Fecsa et al.; who analyzed lead dose-dependent effects for 18 lead exposed individuals and 12 normal volunteers [21]. Jasim et al. also reported a decrease of ALAD activity in battery manufacturing factory workers compared to non-exposed group; furthermore, this decrease became even more evident with increased duration of exposure [28]. The levels of lead in blood were 13.15 μg/dL in the control group, and more than 34.3 μg/dL in the exposed workers, respectively. In India, children residing in urban zones showed a negative correlation ($p < 0.001$) between blood lead levels (mean 11.8 $\pm$ 11.96 μg/dL) and ALAD activity [30].

Recent findings have suggested that ALAD inhibition may occur at low levels of lead in blood. Ahamed et al. reported a significant negative correlation between blood lead levels and ALAD activity in children with blood lead concentration lower than 10 μg/dL [15]. Moreover, Sakai and Morita considered that the threshold value of blood lead for ALAD inhibition is around 5 μg/dL [23]. Nevertheless, Martínez et al. did not find inhibition of enzymatic ALAD activity in children from Argentina, with mean blood lead of 2.58 $\pm$ 0.30 μg/dL [13].

Blood lead levels in our study were lower than in some prior studies on blood lead and ALAD activity [12,13,15,22,23,29,30]. Nevertheless, we observed a significant association between blood lead and ALAD activity at blood lead levels of 2.2 μg/dL, well below the CDC recommended level of 5 μg/dL for children and pregnant women [31]. To our knowledge, a similar result has not yet been reported in the literature.

It is well established that ALAD inhibition results in an increase of δ-ALA levels in blood, which can intensify oxidative stress and release iron from proteins such as ferritin [32]. For that reason, some authors have considered that decrease in ALAD activity has the potential to be used as an indicator of oxidative stress [32–34]. On the other hand, pregnancy is a condition that increases susceptibility to oxidative stress because of the mitochondria-rich placenta. During pregnancy, lipid peroxidation increases due to mitochondrial activity and hormone synthesis in placenta. Iron, which is abundant in the placenta, is important in the production of free radicals, and subjects the fetus to oxidative stress [35].

Importantly, our results also show that a small percent of pregnant women have blood lead concentrations above 5 μg/dL. Similar results were reported in a previous study carried out in Durango, Mexico [36]. A study conducted in Argentina, Mexico and Uruguay estimated 316,703 individuals in these countries are at risk of lead exposure, approximately 0.19% of the total population of all three countries. Of this population, 80,021 were women at childbearing age [37].

Researchers have documented that women with BLLs between 5–10 μg/dL have more probability of having a miscarriage compared to those with BLLs below 5 μg/dL [4]. It is thus necessary to identify and reduce the sources of exposure for these women. Recent research suggested a low threshold for the effect of maternal blood lead on birth outcomes, and recommended that exposure to lead during pregnancy should be kept as low as possible to minimize adverse outcomes [38]. Therefore, the growing evidence regarding the association between low levels of lead in blood and adverse pregnancy outcomes should be taken into account in the development of prevention politics.

We recognized some limitations in our study. In Figure 1 samples with blood lead between 5 and 10 μg/dL show quite a dispersion, but even in this segment the correlation is negative. In contrast, samples with blood lead below 2.2 μg/dL showed a slight increase of ALAD activity. It is well established that ALAD activity is specifically inhibited by lead at concentrations between 5 and 50 μg/dL [9]. In spite of this, significant correlations were observed only in the fourth quartile (BLL >2.19 μg/dL). In the other hand, we did not evaluate some biomarkers of oxidative stress that may be associated with blood lead [39], which could have resulted in uncontrolled confounding. Alcohol consumption may affect ALAD activity, but it was not considered because only a few women recognized they had this habit. Nonetheless, to our knowledge, this is the first study which has analyzed the relationship between blood lead levels and ALAD activity in Mexican pregnant women. Moreover, in the revised literature, there is no such data evaluating the effect of lead exposure on enzymatic ALAD activity in pregnant women, who constitute one of the most vulnerable sections of the population.

5. Conclusions

In summary, the results of our study suggest that even very low lead exposure may cause a decrease of ALAD activity, at least in pregnant women. We propose that ALAD inhibition may occur at very low levels of lead in blood due to lead exposure and pregnancy conditions.

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ARTÍCULO ORIGINAL

Relación heterófilo/linfocito, frecuencia espontánea de eritrocitos micronucleados y prolongaciones nucleares en el ganso nevado (*Chen caerulescens*): Una propuesta como posible biomonitor de estrés y genotóxicos ambientales

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Resumen

Existen organismos silvestres que son altamente vulnerables ante estresores antropogénicos y naturales, estos organismos pueden ser de utilidad para evaluar la salud ambiental mediante diferentes indicadores confiables, sencillos, rápidos, económicos para determinar y si es posible reducir estos efectos negativos. En este estudio proponemos al ganso nevado (*Chen caerulescens)* como posible biomonitor de estrés y genotóxicos ambientales mediante la relación heterófilo/linfocito (H/L), la prueba de micronúcleos (EMN), prolongaciones nucleares en eritrocitos (EPN) y eritrocitos policromáticos (EPC). Durante la temporada de caza (2012-2013) en el humedal de Málaga, Durango, México, colectamos 18 organismos. Los eritrocitos fueron las células más abundantes, de núcleo y morfología elípticos, de tamaño 12.68 ± 0.89 µm. Observamos heterófilos (11.07±1.32 µm), eosinófilos (9.67±1.26 µm), basófilos (5.75 ± 0.78 µm), monocitos (10.49±1.36 µm) y linfocitos (6.53±1.0 µm). No identificamos alteración en las proporciones ni en la morfología de leucocitos, sin embargo, es necesario un mayor número de organismos para establecer los parámetros sanguíneos de base normal para esta especie. La relación H/L fue de 0.41 ± 0.11 este valor es similar a lo reportado para esta y otras especies de aves consideradas como sanas. Establecimos la frecuencia basal de EMN (2.6±1.45), EPN (249.2 ± 89.74) y EPC (156.5 ± 50). El ganso nevado es un organismo que se perfila como buen candidato a biomonitor ambiental debido a la frecuencia basal de su relación H/L, EMN, EPN y EPC, pero debe probarse en condiciones estandarizadas y a través de estudios en zonas con y sin contaminación.

Palabras clave: Biomonitor, *Chen caerulescens*, eritrocitos micronucleados, eritrocitos con prolongaciones nucleares, índice heterófilo linfocito.

Heterophil to lymphocyte ratio, basal frequency of micronucleated erythrocytes and nuclear extensions in the snow goose (*Chen caerulescens*): A proposal as possible stress and environmental genotoxicity biomonitor

Abstract

There are wild organisms that are highly vulnerable to anthropogenic and natural stressors, these organisms may be useful to assess environmental health through various reliable, simple, rapid, inexpensive indicators and to determine and if possible reduce these negative effects. In this study we propose that the snow goose (*Chen caerulescens*) to be tested as stress and environmental biomonitor of genotoxicity, by heterophil/lymphocyte (H/L) ratio, micronucleus test (MNE), nuclear protrusions in erythrocytes (NPE) and polichromatic erythrocytes (PCE). During the hunting season (2012-2013) in the wetland of Malaga, Durango, Mexico, 18 organisms were collected. Erythrocytes were the most abundant cells with elliptical morphology and nucleus, size of 12.68 ± 0.89, regular contour and nucleus-cytoplasm ratio 1:2. We observed heterophils (11.07 ± 1.32 µm), eosinophils (9.67 ± 1.26 µm), basophils (5.75 ± 0.78 µm), monocytes (10.49 ± 1.36 µm) and lymphocytes (6.53 ± 1.0 µm). No alteration in the proportions or morphology of leukocytes was identified, however, a greater number of organisms are required to establish normal blood parameters basis for this species. H/L ratio was 0.41±0.11 this value is similar to that reported for this and other bird species classified as healthy. MNE basal rate (2.6 ± 1.45), NPE (249.2 ± 89.74) and PCE (156.5 ± 50) was established. The snow goose is an organism that is emerging as a good candidate for environmental biomonitor due to basal frequency of H/L, MNE, NPE and PCE, but must be tested under standardized conditions and through studies in areas with and without pollution.

Keywords: Biomonitor, *Chen caerulescens,* micronucleated erythrocytes, erythrocytes with nuclear protrusions, heterophil/lymphocyte ratio.

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Introducción

La contaminación del ambiente por causas antropogénicas es un problema mundial que va en incremento (Botello *et al.* 2015), sus consecuencias son múltiples y diversas, cada una de ellas digna de soluciones inmediatas. El daño por agentes físicos o químicos en el material genético como los cromosomas o el ADN se estudia en términos de genotoxicidad (Newman 2010), el daño genotóxico habitualmente es silencioso, pasa inadvertido y se puede expresar como mutagénico, teratogénico o cancerígeno (Mathews 2015).

Los resultados suelen ser muy dramáticos ya que ponen en riesgo la vida del organismo e incluso sitúan en peligro de extinción a la especie. Por ello es importante contar con biomonitores e implementar sistemas de monitoreo que brinden información oportuna. Los métodos para evaluar el riesgo o protección a genotóxicos con frecuencia son costosos, complicados e invasivos. Ante este panorama la técnica de micronúcleos (MN) que permite la detección de genotóxicos, mutagénicos, teratogénicos e inestabilidad genómica, ofrece una gran oportunidad. Esta técnica es una prueba precisa, confiable, mínimamente invasiva, no se requiere de cultivos celulares, es rápida, sencilla y relativamente económica. Además, a estas cualidades se suman la versatilidad de aplicación en diversos organismos, tejidos y modelos, y no requiere de instalaciones especiales ni del sacrificio de los organismos (Zúñiga *et al.* 1996, Torres-Bugarín *et al*. 2014).

Biomonitores y biomarcadores

La cuantificación de contaminantes en suelo, agua o aire no permite evaluar la biotransformación, la biodisponibilidad o los efectos sobre los organismos, por eso se complementa con la *biomonitorización* que facilita la medición cualitativa o cuantitativa de exógenos o sus metabolitos en los organismos o en el ecosistema (Capó 2007). Biomonitores pueden considerarse los organismos vivos sensibles a cambios ambientales (Markert *et al*. 2003, Capó 2007, Needham 2007) o bien especies acumuladoras de contaminantes a niveles que permiten ser evidenciados antes que en muestras abióticas (Spahn y Sherry 1999). Un buen monitor debe tener representatividad espacial y ecológica, disponibilidad y accesibilidad, sensibilidad, longevidad de la especie y reproducibilidad (Tataruch y Kierdorf 2003).

Específicamente, el grupo de aves es muy diverso, sus integrantes habitan en múltiples ambientes, sus respuestas a estresores naturales y antropogénicos son muy variadas (Mallory *et al*. 2010, Páez-Osuna y Osuna-Martínez 2011, Sheperd y Somers 2012). En particular, las aves acuáticas son candidatas a ser biomonitores por su amplia distribución, su abundancia y representatividad en el ecosistema (Markert *et al*. 2003, Mallory *et al*. 2010). Éstas se desenvuelven en diferentes partes de las redes tróficas y sus respuestas a los cambios ambientales pueden ir desde ligeros efectos fisiológicos hasta deformaciones o muerte (Hoffman *et al*.1996, Mallory *et al*. 2010). Adicionalmente existe la necesidad de realizar estudios que permitan la conservación, protección y uso adecuado de las aves acuáticas que migran cada año hacia centro y Sudamérica (Barba 2006). En este grupo encontramos al ganso nevado (*Chen caerulescens*), es una ave anseriforme de la familia Anatidae, la cual se reproduce en el Ártico, en áreas pantanosas de la tundra en Canadá, Alaska, Groenlandia, además de la isla Wrangell del extremo nororiental de Siberia, y migra al sur para sus estancias de residencia tropical en en Japón y a lo largo de la parte central y meridional de Norteamérica y centro-norte de México, su hábitat invernal son los pantanos, campos de cultivo, praderas, charcos y bahías (Heyland 2000, Peterson y Chalif 2008). Se alimenta de raíces, bulbos, brotes y hojas (BirdLife International 2015). Esta ave posee características ideales para ser considerada biomonitor, entre ellas su situación poblacional estable, su amplia distribución geográfica, su dieta, el tipo de hábitat en donde se alimenta y por que recorre grandes distancias (Bellrose 1976, Heyland 2000).

El riesgo de deterioro de la salud de cualquier organismo puede ser evaluado mediante marcadores biológicos conocidos como *biomarcadores*, éstos pueden ser alteraciones celulares, bioquímicas o moleculares, medibles en tejidos, células, o fluidos, o bien características biológicas que puedan ser cuantificadas en procesos fisiológicos, patológicos, respuestas farmacológicas, evaluación experimental, medición de riesgo ambiental y epidemiológico (Mayeux 2004). Dentro de los biomarcadores se identifican tres grupos: 1) de exposición, 2) de efecto y 3) de susceptibilidad (Arango 2012).

Eritrocitos micronucleados (EMN) como biomarcador de genotóxicos

En función de las características necesarias para la elección de un buen biomonitor mediante la técnica de micronúcleos (MN) en sangre periférica, se considera que las aves son un grupo adecuado debido a que tienen un bazo pequeño y pobre en elementos reticuloendoteliales, eritropoyesis muy activa, incluso más alta que los mamíferos, el recambio celular es aún mayor y la relación citoplasma-núcleo por lo general es 1:2 (Aschoff 1928, Ramírez-Muñoz *et al*. 1999, Udroiu 2006, Gómez-Meda *et al*. 2008, Clark *et al*. 2009,Weiss y Wardrop

2010, Sheperd y Somers 2012). Un MN (en hematología mejor conocido como cuerpo de Howell-Jolly) puede ser un fragmento o un cromosoma completo que durante la división celular se perdió debido a un daño en el uso mitótico (daño aneuploidogénico) o bien a la fractura de un cromosoma (daño clastogénico). En eritrocitos los MN se forman en médula ósea, luego se liberan en sangre periférica y si el sistema reticuloendotelial es eficiente éste será retirado en muy poco tiempo, de lo contrario permanecerá por un periodo prolongado en sangre periférica, y es cuando pueden ser visualizados más fácilmente (Ramírez-Muñoz *et al.* 1999). Un reticulocito es un eritrocito joven (eritrocito policromático - EPC) que aún contiene RNA y son indicadores de eritropoyesis o citotoxicidad, un EPC en 24 h se transforma en eritrocito normocromático, el cual, en las aves, permanece en circulación durante 25-28 días. Por tanto si se detecta frecuencia mayor de 3 eritrocitos micronucleados en 10 000 eritrocitos totales (EMN) se considera daño crónico y si son EPC micronucleados (EPCMN) muestra daño ocurrido en menos de 24 h (Schmid 1975, Zuñiga *et al.* 2001).

Conteo diferencial leucocitario

El principal componente del sistema inmunitario de los vertebrados está mediado por los leucocitos y en las aves existen cinco tipos: heterófilos, eosinófilos, basófilos, linfocitos y monocitos (Gershwin *et al.* 1985). El aumento o disminución de un determinado tipo de leucocito, además de reflejar enfermedades, también responden al grado de estrés a que está sometido un organismo, incluso estrés por malnutrición y pérdida de peso significativa o un ejercicio físico extremo (Gershwin *et al.* 1985, Maxwell y Robertson 1998). Los eventos estresantes incrementan los heterófilos y disminuyen los linfocitos, razón por la cual la proporción heterófilos/linfocitos (H/L) es aceptada como una medida de respuesta fisiológica de inmunosupresión y estrés (Davis *et al.* 2008, Cirule *et al.* 2012, Genovese *et al.* 2013).

El propósito de este estudio fue evaluar la relación H/L, frecuencia de EMN, EPN y EPC en sangre del ganso nevado (*Chen caerulescens*) para generar valores de referencia y evaluar su factibilidad como biomonitor ecotoxicológico.

Métodos

Descripción del área de estudio

El sitio de colecta se ubica al noroeste de la ciudad de Durango (24°07′00″ N, 104°30´20″ O y altitud de 1 861 m), forma parte de la Unidad de Manejo Ambiental (UMA) del Ejido "El Arenal" (registro SEMARNAT-UMA-EX0336-DGO), con superficie total de 5 246 ha, el uso de suelo es agropecuario. La colecta del ganso nevado (*Chen caerulescens)* la realizamos en coordinación con los grupos de cazadores que visitaron la UMA durante la temporada de caza de diciembre de 2012 a enero de 2013. Por tanto, la toma de muestras la realizamos en dos momentos diferentes, una en diciembre (6 organismos) y otra en enero (12 ejemplares), esto significa que las aves arribaron a México aproximadamente entre 40 y 80 días antes de tomar las muestras. En el sitio de colecta pesamos cada ave (bascula Torrey Modelo LEQ 10/20, con precisión de 0.02 kg), definimos la edad como joven o adulto según la coloración del plumaje y presencia de muda o estado de las plumas primarias. C. *caerulescens* es una especie cuyas hembras y machos tienen una fisonomía similar por lo que los sexamos mediante necropsia. A cada individuo le tomamos una muestra de 0.5 mL de sangre y realizamos dos frotis de cada ejemplar sobre un portaobjetos previamente limpio, desengrasado y codificado, dejamos secar al aire libre y fijamos en etanol al 80% durante 10 minutos y posteriormente realizamos la tinción.

Los frotis sanguíneos los teñimos con solución Wright-Giemsa durante 5 minutos, luego agregamos 10 gotas de tiosulfato de sodio y dejamos reposar durante 5 minutos, después lavamos las muestras con agua destilada y los secamos al aire libre. Para el conteo diferencial leucocitario y posteriormente obtener el índice H/L contabilizamos 200 leucocitos por organismo con el microscopio 100x (ZEISS modelo Axiostar plus) (Maxwell y Robertson 1998, Ontiveros-García 2006).

El análisis de eritrocitos micronucleados (EMN) y prolongaciones nucleares en eritrocitos (EPN) lo realizamos con dos tinciones Wright-Giemsa y anaranjado de acridina (tinción altamente específica para ácidos nucleicos, que tiñe DNA verde limón y el RNA anaranjado), para el análisis con la primer tinción utilizamos el mismo frotis para el diferencial leucocitario, y para el análisis con anaranjado de acridina utilizamos el segundo frotis de cada individuo. Los frotis fijados y teñidos con anaranjado de acridina los analizamos con un microscopio equipado con fluorescencia (100x) (ZEISS modelo Axiostar plus). Por cada tinción cuantificamos 10 000 eritrocitos totales en los que identificamos la frecuencia de EMN y EPN, y contabilizamos la frecuencia de EPC en 1000 eritrocitos totales (Gómez *et al.* 2006).

Análisis estadístico

Realizamos el análisis de normalidad de los datos (Shapiro-Wilk) e igualdad de varianzas (Levene), y calculamos los es-

tadísticos más relevantes (promedio, desviación estándar, rangos) para las variables de estudio como peso de las aves (kg), diámetro mayor y menor (µm) de las células sanguíneas, frecuencia de leucocitos y en eritrocitos la frecuencia de EMN, EPN y EPC. Utilizamos la prueba de Tukey (α=0.01) para realizar las comparaciones entre las medias de los estadísticos evaluados para cada sexo. Para todos los análisis usamos el programa estadístico NCSS (Hintze 2001).

Resultados

Durante la temporada de caza 2012-2013 en el humedal de Málaga, Durango, México, colectamos 18 organismos adultos, de los cuales cinco fueron hembras, seis machos y siete sin sexar porque implicaba hacer necropsia y los cazadores deseaban conservar las aves. El peso promedio fue de 1.867 ± 0.261 kg, con rango de 1.478 a 2.354 kg, sin diferencias estadísticas (p= 0.287) entre sexos.

Diferencial leucocitario y relación H/L

El análisis del frotis sanguíneo de *Chen caerulescens* nos permitió establecer que los eritrocitos normales fueron las células más abundantes, su diámetro mayor fue de 12.68 ± 0.89 µm (n= 700, mínimo 11 µm y máximo 15 µm), el diámetro menor fue de 6.41 ± 0.3 µm (n= 700, mínimo de 5 µm y máximo 9 µm), el núcleo de estas células es elíptico de contornos regulares, con diámetro mayor de 6.44 ± 0.14 µm, por tanto la relación citoplasma-núcleo es de 1:2. Dentro de los leucocitos observamos cinco tipos celulares: heterófilos, los más abundantes (11.07 ± 1.32, 8-13.5 µm), linfocitos (6.53 ± 1.0 µm; 4.9-9 µm), eosinófilos (9.67 ± 1.26 µm; 6.9-12.3 µm), monocitos (10.49 ± 1.36; 7.5- 13.2 µm), basófilos (5.75 ± 0.78; 4.1 - 7.3 µm) (Cuadro 1). La proporción H/L fue de 0.41 ± 0.11, no hubo diferencias significativas (p>0.01) entre frecuencia de leucocitos y relación H/L (p=0.4825) para organismos de diferente sexo.

Frecuencia de EPC, EMN y EPN

La frecuencia basal de EMN fue de 2.63 ±1.45 con rango de 0-5. La frecuencia basal de EPN fue de 249.25 ± 89.74 con rango de 95 - 458 y en cuanto a la de EPC fue de 156.56 ± 50.01 con rango de 71- 227 (Figuras 1, 2 y 3).

Discusión

Algunos valores hemáticos de los gansos se modifican como resultado de cambios en su estado fisiológico asociados a su

Cuadro 1. Diferencial leucocitario, frecuencia de micronúcleos y prolongaciones nucleares en eritrocitos en el ganso nevado (*Chen caerulescens*).

	Peso	Granulocitos					Relación Het/Lin	Eritrocitos		
	g	Mon	Eos	Bas	Het	Lin		EPC	EMN	EPN
Hembras *n=5*										
Media	1707.60	8.75	8.50	10.75	21.75	51.75	0.42	191.00	2.75	298.00
DE	213.49	2.62	1.29	2.06	4.42	2.98	0.009	28.72	1.26	40.63
Machos *n=6*										
Media	1915.00	9.50	6.66	9.66	19.66	56.00	0.36	164.83	2.83	208.67
DE	278.65	4.72	2.16	3.14	4.67	5.58	0.12	42.30	1.83	94.76
Aves sin sexar *n=7*										
Media.	1939.71	7.33	6.66	11.83	22.5	52.33	0.44	125.33	2.33	257.33
DE	262.52	1.86	1.96	4.49	4.41	8.04	0.12	54.93	1.37	101.17
General *n=18*										
Media	1867.00	8.50	7.12	10.75	21.25	53.56	0.41	156.56	2.63	249.25
DE	261.44	3.30	1.96	3.43	4.40	6.13	0.11	50.01	1.45	89.74

g gramos; Diferencial leucocitario en 200 leucocitos (Mon-Monocitos; Eos-Eosinófilos; Bas-Basófilos; Het-Heterófilos; Lin-Linfocitos). En 1000 eritrocitos totales (ET) (EPC- Eritrocitos policromáticos). En 10 000 ET- (EMN- Eritrocitos micronucleados; EPN- eritrocitos con prolongaciones nucleares).

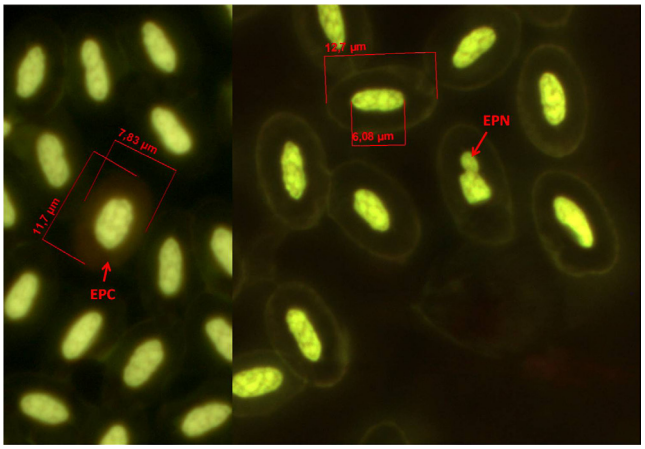

Figura 1. Sangre de *Chen caerulescens,* Eritrocito policromático (EPC), Eritrocito con prolongación nuclear (EPN), Tinción: anaranjado de acridina (100x).

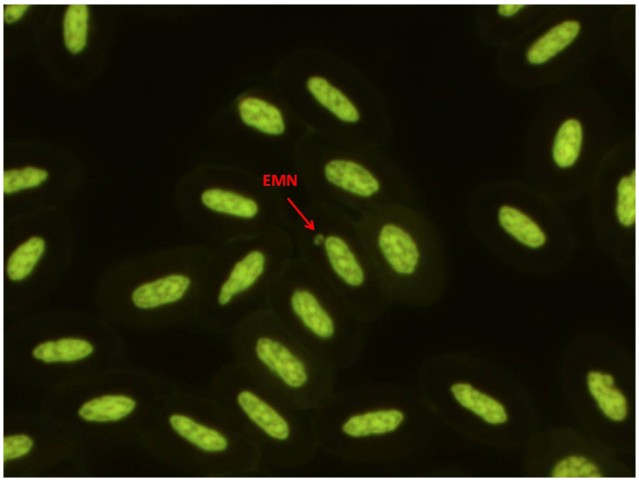

Figura 2. Sangre de *Chen caerulescens,* Eritrocito micronucleado (EMN), Tinción: anaranjado de acridina (100x).

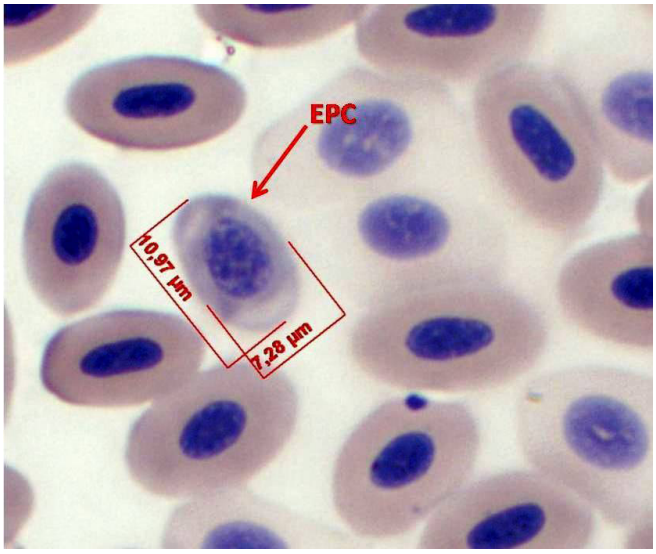

Figura 3. Sangre de *Chen caerulescens,* Eritrocito policromático (EPC), Tinción Wrigth-Giemsa (100x).

comportamiento migratorio (Williams y Trainer 1971), esto podría observarse en fechas cercanas (octubre y noviembre) a su llegada a los sitios de residencia no reproductiva en México. La toma de muestras la realizamos aproximadamente entre 40 y 80 días después del arribo de las aves, y debido a que las células hemáticas viven en promedio 28 días, podemos entender que nuestros resultados reflejan los factores ambientales prevalecientes en el sito de colecta. Sin embargo, sería interesante determinar la frecuencia leucocitaria, el índice H/L, así como la frecuencia de EMN, EPN y EPC al momento del arribo y por supuesto antes de la migración en su lugares de origen, incluso evaluar el comportamiento de estos parámetros de acuerdo con su edad aproximada, peso y sexo.

Diferencial leucocitario y relación H/L

El conteo para el diferencial leucocitario se recomienda entre 100 y 200 células, en este caso cuantificamos 200 leucocitos por organismo, y está descrito que los conteos leucocitarios se modifican en condiciones de estrés, enfermedades y otros factores (Davis *et al.* 2008, Cirule *et al.* 2012, Genovese *et al.* 2013). En *Chen caerulescens* no identificamos alteraciones en las proporciones ni en la morfología de leucocitos descrita para otras especies de gansos y no encontramos hemoparásitos. Sin embargo, reconocemos que para establecer los parámetros sanguíneos de base normal para una especie, es necesario una muestra de mayor tamaño que represente una amplia gama demográfica de los animales, por ello se necesitan más evaluaciones para esta especie (Charles-Smith *et al.* 2014). Por otro lado, si bien el índice H/L no debe de ser el único parámetro para valorar la salud y condición inmunológica de las aves, reconocemos que es un buen indicador de estresores agudos y crónicos (Álvarez 2010, Cotter 2015). Las aves en respuesta a factores de estrés aumentan la actividad del eje adrenal hipotálamo-hipófisis, dando lugar a concentraciones elevadas de glucocorticoides, lo que induce a su vez aumento de la frecuencia de heterófilos (células equivalentes a los neutrófilos de los humanos), y disminución en los linfocitos, a esta circunstancia se define como elevado índice H/L (Meer y Oers 2015). Los heterófilos son la primera resistencia natural contra infecciones bacterianas y otros agentes (Gross y Siegel 1983, Cirule *et al.* 2012) entonces este parámetro se correlaciona con inmunosupresión, con bajas tasas de crecimiento, supervivencia, niveles elevados de glucocorticoides y estrés.

El índice H/L en *C. caerulescens* fue de 0.41 ± 0.11, dato similar al descrito por Williams y Trainer (1971) H/L 0.31 y al de otras especies calificadas como sanas (Cuadro 2). El playero

Cuadro 2. Relación heterófilo/linfocito (H/L) en diferentes especies de aves.

Orden - Familia	Especie	Características	Índice H/L	Referencia
	Chen caerulescens	Migratoria	0.31	Williams y Trainer (1971)
Anseriformes Anatidae	*Branta canadensis*	Residente	Adulto: 0.34 ± 0.3 Juvenil: 0.17 ± 0.1 Total: 0.32 ± 0.3	Charles-Smith *et al.*(2014)
Galliformes Pasianidae	*Gallus gallus*	Doméstica	0.48 Semanas en aviarios 18 / 0.25 77 / 0.33	Branton *et al*. (1997) Cotter (2015)
Columbiformes Columbidae	*Zenaida asiática*	Migratoria	1.39	Pérez *et al*. (2008)
Apodiformes Trochilidae	*Colibri sp.*	Residente	0.9	Matta-Camacho *et al.* (2014)
Charadriiformes Scolopacidae	*Calidris canutus rufa*	Migratoria	Escala trófica: 0.4 ± 0.1 Invernada 1.4 ± 0.1	D'amico (2011)
Laridae	*Larus argentatus*	Migratoria	0.48	Fox *et al.* (2007)
Falconiformes Falconidae	*Falco cenchroides*	Residente	0.39	Clark *et al*. (2009)
Passeriformes Laniidae	*Lanius ludovicianus*	Residente	♀ 0.88 ± 1.39 ♂ 1.04 ± 0.90	Álvarez (2010)
Páridae	*Parus major*	Residente	0.29	Hauptmanova *et al.* (2002)
Corvidae	*Corvus coronoides*	Residente	0.39	Clark *et al*. (2009)

ártico (*Calidris canutus rufa*) es un ave migratoria que habita el norte del continente americano cuyo índice H/L es similar en su época reproductiva (0.4 ± 0.1), sin embargo este índice se modifica durante su estancia invernal (1.4 ± 0.1) (D'amico 2011).

Frecuencia de EMN

La formación de MN es un marcador ampliamente explotado para evaluar agentes genotóxicos, pues estas estructuras pueden ser identificadas sencillamente en cualquier célula somática o germinal que se divida. Específicamente en sangre es factible su uso por la facilidad para obtener la muestra, a la abundancia de células y no se arriesga la vida del organismo. En mamíferos los eritrocitos son anucleados, lo que permite la detección de los MN, no obstante en ellos es fundamental considerar la funcionalidad del bazo para que un organismo pueda ser utilizado como biomonitor de genotóxicos, pues en muchos mamíferos este órgano es el principal responsable de eliminar glóbulos rojos viejos, anómalos o que se encuentran en mal estado, esto dependiendo de si el bazo es sinusoidal (altamente vascularizado) o no sinusoidal (Udroiu 2006). En cambio, en organismos no mamíferos el bazo no compromete la prueba de MN ya que este órgano no es sinusoidal y posee vénulas fenestradas que permiten que sus eritrocitos nucleados pasen fácilmente (Udroiu 2006). Particularmente en las aves, el bazo es un órgano secundario linfoide muy pequeño, de forma redondeada, se localiza al costado derecho, entre el proventrículo y el estómago muscular; sus funciones principales son captar los antígenos circulantes en la sangre, activar los macrófagos y desencadenar la producción de células plasmáticas específicas, y probablemente la función de eliminación de eritrocitos anómalos o con inclusiones, como los MN, quede

relegado al resto del sistema reticuloendotelial como serían macrófagos libres, ganglios o hígado (Hernández 1998).

En este caso el ganso nevado presentó 2.63 ±1.45; rango de 0-5 EMN/10 000 (Cuadro 1), entonces sugiere que es un organismo con bazo no sinusoidal y que el resto del sistema retículo endotelial no es lo suficientemente eficiente como para remover de la circulación todos los eritrocitos viejos, anómalos o con inclusiones, por lo tanto podría ser un organismo ideal para ser utilizado con fines de monitoreo ambiental nacional y trasnacional. En un estudio en el que incluyeron mamíferos, reptiles y aves, describen ausencia de EMN en *C. caerulescens* (Cuadro 3), no obstante en esa ocasión sólo se utilizaron muestras de aves en cautiverio y dos organismos en vida silvestre (Zúñiga *et al*. 2001). En ese mismo estudio se proponen como monitores aquellos organismos que presentaron más de 3.5 EMN/10 000 eritrocitos totales y consideran que la edad del organismo es importante, ya que al menos en la ardilla gris la frecuencia de EMN espontáneos es más abundante, dato que posteriormente corroboran mediante un estudio experimental (Zúñiga *et al*. 2001). Por otro lado, en el perico atolero (*Aratinga canicularis*) la frecuencia de EMN fue 3/10 000, dato muy similar al del ganso nevado, y al ser expuesto a genotóxicos la frecuencia se incrementó hasta 6/10,000 EMN (Gómez *et al*. 2006). Es recomendable realizar la exploración de MN en las diferentes familias de aves y detectar aquellas que posean un sistema retículo endotelial deficiente para poder identificar fácilmente los EMN espontáneos o inducidos y poder proponer biomonitores de agentes genotóxicos ambientales.

Frecuencia de EPN

Los eritrocitos de las aves son típicamente elípticos con núcleo central, pero con frecuencia la morfología nuclear muestra variantes, como núcleos simétrica y asimétricamente constreñidos, bilobulados, binucleados y microlobulados (Clark *et al*. 2009). Al igual que los MN, estas alteraciones son consideradas como potenciales marcadores de genotoxicidad (Serrano-García y Montero-Montoya 2001, Gómez *et al*. 2006); por ejemplo, en el perico atolero (*A. canicularis)*, se le administró una inyección intracoelomica de 4 mg/kg mitomicina-C, y observamos incremento paralelo tanto en los EMN (0 horas 2.89± 2.4/72 horas 6.09 ± 2.5) como en los EPN (0 horas 8.89 ± 6.8/72 horas 14.29±4.4), por lo tanto ambos parámetros deben ser considerados en estudios de toxicología genética ambiental (Gómez *et al*. 2006).

En el ganso nevado el promedio de EPN fue de 249.25 ± 89.74 /10 000 ET, pero no existe información previa que permita comparar nuestros resultados. Sin embargo, el promedio de EPN obtenido es superior al compararlo con los valores encontrados en otras especies de aves (Cuadro 4), lo que sugiere que este biomarcador podría ser muy sensible en estudios toxicológicos. En nuestros resultados el número de EMN fue menor al número de EPN, lo cual es consistente con los experimentos realizados con la especie *A. canicularis*, y que la frecuencia de estas dos anormalidades se incrementa de forma paralela (Gómez *et al.* 2006). En el estudio con el págalo antártico (*Catharacta maccormicki)* la cantidad de EPN fue diez veces superior a la de EMN (Krusa y Bezrukov 2007). Este comportamiento sugiere que la formación de EPN puede deberse a un espectro de factores más amplio que las que originan la formación de los EMN (Gómez *et al.* 2006). Una consideración importante que se observa en los estudios citados (Cuadro 4) es que los valores de EPN tuvieron una gran variabilidad ocasionada posiblemente por tratarse de aves de diferente sexo, edad y peso, lo cual se relaciona con la madurez del sistema retículo endotelial de las aves y a su capacidad para remover células dañadas de la circulación.

Eritrocitos policromáticos con micronúcleos y prolongaciones nucleares

En aves el periodo de vida de un eritrocito es de 25 a 28 días (Dukes y Swenson 1981), promedio menor al de los mamíferos que es de 120 días aproximadamente, esto confiere la posibi-

Cuadro 3. Eritrocitos micronucleados (EMN)/10 000 ET en diferentes especies de aves.

Especie	Promedio ±DE	n	Referencia
Chen caerulescens	0	2	Zuñiga *et al.* (2001)
Anas platyrhynchos	0.20	8	Zuñiga *et al.* (2001)
Anas clypeata	1.45±2.06	20	Ross-Muñoz (2011)
Anas acuta	1.71± 1.70	22	Ross-Muñoz (2011)
Aratinga canicularis	3.0 ± 3.6	7	Gómez *et al.* (2006)
Catharacta maccormicki	0.07 ± 0.3	162	Krusa y Bezrukov (2007)

Cuadro 4. Prolongaciones nucleares (EPN) descritos en diferentes especies de aves.

Especie	N	Promedio ±DE 10 000 ET	Referencia
Anas clypeata	20	47.75±50.89	Ross-Muñoz 2011
Anas acuta	22	20.81±1.76	Ross-Muñoz 2011
Aratinga canicularis	7	12.8 ± 21.4	Gómez *et al.* 2006
Catharacta maccormicki	162	0.71 ± 1.02	Krusa y Bezrukov 2007

lidad de realizar estudios a corto plazo. No obstante es necesario identificar los posibles efectos genotóxicos en plazos mucho más cortos, y es cuando son útiles los valores de EPC con micronúcleos (EPCMN). Pero no todas las especies animales tienen EPC, afortunadamente el ganso nevado tiene muy buenas frecuencias de EPC 156.56 ± 50.01/1000 eritrocitos totales. No se observaron EPCMN y EPCPN en los 16 organismos analizados. La presencia de estas anormalidades se sugiere que sucede durante la exposición crónica (niveles elevados de xenobióticos y periodos de tiempo prolongados) a ciertas sustancias contaminantes (Navarro y Benítez 1995).

El ganso nevado (*Chen caerulescens*) es un organismo que puede ser considerado como buen candidato a biomonitor ambiental debido a la frecuencia basal de su relación H/L, EMN, EPN y EPC, pero debe probarse en condiciones estandarizadas y a través de estudios en zonas con y sin contaminación. De manera complementaria se requiere hacer estas mismas determinaciones en especies de aves antes de la migración, así como a individuos de diferentes edades.

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Frontotemporal Lobe Degeneration as Origin of Scans Without Evidence of Dopaminergic Deficit

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The term scans without evidence of dopaminergic deficit (SWEDD) can be associated with any patient diagnosed at first with Parkinson's disease but with a negative dopamine transporter-single photon emission computed tomography (DaTSPECT), which does not confirm the presynaptic dopaminergic deficiency. Therefore, an alternative diagnosis should be sought to support parkinsonism as a clinical diagnosis. Parkinsonism is a well-known manifestation of frontotemporal lobar degeneration (FTLD), particularly frequent in those with positive DaTSPECT. Here, we reinforce previous observations that parkinsonism can be present in FTLD patients with negative DaTSPECT and therefore, FTLD may account for a percentage of patients with SWEDD. We gather the clinical observations supporting this hypothesis and describe a case report illustrating this idea. Studies suggest the result of DaTSPECT in FTLD may depend on the neuropathology and clinical subtype. However, most studies do not provide a clinical description of the clinical subtype or pathological features making the association between subtypes of FTLD and DaTSPECT results impossible at the moment. Further studies correlating clinical, neuropsychological, neuroimaging, genetic, and pathology findings are needed to better understand parkinsonism in FTLD.

Keywords: scans without evidence of dopaminergic deficit, parkinsonism, dopamine transporter-single, photon emission computed tomography, presynaptic dopaminergic deficiency, frontotemporal lobar degeneration

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder. It is characterized by progressive degeneration of dopaminergic neurons in the *pars compacta* of the substantia nigra and the loss of nerve terminals in the basal ganglia structures (1, 2). The dopaminergic system is one of the most studied neurochemical systems because damage to nigrostriatal neurons is the most critical component in the pathophysiology of PD (1, 3). Clinically it is manifested by the so-called "parkinsonian syndrome," consisting of extrapyramidal signs, including bradykinesia and at least one of the following: 4–6 Hz rest tremor, muscular rigidity and postural instability. The term "parkinsonism"

encompass some nosologic entities, besides PD, which are grouped by their shared clinical features but are separated by their different pathologies.

The role of nigrostriatal dopamine deficits in PD has been firmly established (4–6). In recent years, dopamine transporter-single photon emission computed tomography, named DaTSPECT, has been used to detect degeneration of presynaptic dopamine receptors in the nigrostriatal structures (6, 7). The active ingredient of [123I]FP-CIT SPECT is a cocaine analog, 123I-nortropane, labeled: *N*-u-fluoropropyl 2b-carbomethoxy-3b-(4-iodophenyl), also referred to as: ([123I]ioflupane). It binds to striatal presynaptic dopamine transporter (DaT) in animals and humans and helps visualize indirectly these neurons with SPECT. DaT is located on the plasma membrane, mainly of nerve terminals of dopaminergic neurons in the brain, particularly in the globus pallidus, cingulate cortex, amygdala, olfactory tubercle, and midbrain but specially in the striatum and nucleus accumbens. DaT reuptakes dopamine from the synaptic cleft into presynaptic neurons playing an important role in the buffering of this neurotransmitter (8).

Baseline DaT imaging has a very high negative predictive value for degenerative conditions affecting the nigrostriatal pathway. Only a negligible proportion of normal dopaminergic SPECT can be found in atypical parkinsonisms (9). The acronym SWEDDs (scans without evidence of dopaminergic deficits) (10), arose from the clinical trial literature of PD, in which patients were imaged with 18F-dopa PET or DaTSPECT to monitor disease progression, revealing that a substantial proportion of clinically diagnosed cases of PD had regular nuclear medicine scans (4–15%) and were, therefore, designated as SWEDDs (10, 11). Thus, the term SWEDDs can be associated with any patient diagnosed at first with PD but in which subsequent functional imaging did not confirm the presynaptic dopaminergic deficiency, although the use of the term SWEDD is itself controversial (9, 12).

The true etiology of the symptoms experienced by patients with SWEDD remains controversial, and it has been suggested that these individuals may represent a non-PD related movement disorder (10, 13, 14). When patients with SWEDD were initially discovered, it was hypothesized that they might be within a prodromal phase of PD (15). However, subsequent research has demonstrated significant differences between patients with dopamine-deficient scans and patients with SWEDD. Patients with SWEDD lack response to levodopa (16) and do not demonstrate deficits in olfaction as frequently as patients with dopamine-deficient PD (17). Patients with SWEDD also have more significant cardiovascular and thermoregulatory dysfunction, orthostatic hypotension, sleep disturbances, and higher frequencies of daytime sleepiness than dopamine-deficient PD patients (18, 19). Although, patients with SWEDD can present motor features similar to those which are dopamine-deficient (PD), previous longitudinal research suggests that patients with SWEDD do not demonstrate the progression of motor symptoms (20) and continue to have normal DaTSPECTs for up to 4 years after they are initially identified (19, 21, 22). In a 5-year follow-up study of 16 patients with SWEDD, only 2 patients demonstrated reduced dopamine uptake on DaTSPECTs, while 14 remained classified as SWEDD (11). These studies seem to indicate that individuals with SWEDD have a distinctly different disorder than dopamine-deficient PDs (14, 15, 18, 22). Moreover, patients with SWEDD and long-standing parkinsonism exhibit non-motor features that differ from those of patients with PD. SWEDD patients had worse mood and cardiovascular function and better olfactory function than PD patients, but remain similar to patients with SWEDD and alternative final diagnoses (19). In general, the term SWEDD is a useful approach for those patients with slow-progressing parkinsonism, with mild evolution compared with PD. Some frontotemporal lobar degeneration (FTLD) have parkinsonism and a mild clinical course, probably this subgroup could represent a minority percentage of SWEDD (23–26).

In a prospective study of our research group, 30 patients with hard-to-diagnose tremor and normal DaTSPECT were followed for 2 years (27). After the follow-up scan diagnosis was reached for 18 cases. The other 12 patients underwent a second DaTSPECT and were then followed for 12 additional months. After this, the clinical diagnosis was performed again. The final diagnoses included a list of different entities, including neurodegenerative and non-neurodegenerative disorders. However, for six patients diagnosis remained uncertain. Interestingly, these six patients developed cognitive impairment with outstanding frontal features. It was first speculated that although some patients with SWEDDs had, in fact, dystonic tremor or other well-known neurological conditions, it was conceivable that some patients might have suffered from a disorder that had not yet been described. It was speculated that some of these patients might have suffered a neurodegenerative process originated in the frontal cortex spreading to subcortical structures (versus the subcortical → cortical process present in PD). In this article, we present a case report illustrating this possibility and discuss the clinical, neuropsychological, genetics, and neuroimaging findings supporting the observation that FTLD is behind some cases of SWEDD.

CASE REPORT

Written informed consent was obtained from the patient for the publication of this case report. She is a 70-year-old woman who worked as a childcare worker and is now retired. She used to drink 30 g alcohol/day until 10 years ago and she still smokes 5 cigarettes/day. She was diagnosed with depressive syndrome 10 years ago. Currently, she is on Trazodone 100 mg/day and Clonazepam 0.5 mg/day. There are cases of PD (brothers) and depression (mother and maternal grandmother) among her family records.

She first visited neurology clinics 10 years ago with memory loss complaints. On that occasion, cognitive screening tests, blood tests, and CT scan were normal. Complaints were supposed to be in relation with a mood disorder, and no treatment was prescribed. She came again 5 years ago due to impairment of memory. She also complained about unbalanced gait, intentional tremor, and difficulty doing fine tasks. Caregivers referred social, behavioral changes, and personal care difficulties, even when she was still living on her own at that time.

Neurological examination showed hypomimia, mild rigidity in the four limbs, global bradykinesia, and unbalanced gait. There

was a mild and mixed (rest and action) tremor affecting the four limbs. Her Mini-Mental Status Examination reflected deficits in memory, while her performance on the Frontal Assessment Battery was notable for impairment in inhibiting automatic responses, verbal fluency, and Trail making tests A & B. She also demonstrated deficits in the memory and verbal fluency sections of the Seven Minute Screen.

Laboratory tests were all normal. Baseline MRI showed mild diffuse atrophy of the cerebrum together with some small vessel lesions affecting periventricular and semioval white matter. Follow-up MRI (5 years later) showed more intense atrophy in the left frontal and temporal lobes (**Figure 1A**). Tc-99m-HMPAO SPECT showed bilateral but asymmetric hypoperfusion (more on the left side) on frontotemporal lobes (**Figure 1B**). 18F-FDG-PET showed mild to severe hypometabolism on the left frontotemporal lobe junction, and mild hypometabolism on the left fronto-basal left anterior cingulum regions (**Figure 1C**). DaTSPECT was informed as for the normal density of presynaptic dopaminergic uptakers (**Figure 1D**). Some irregularities in the morphology of basal ganglia and increase of cortical uptake were present.

The final diagnosis was frontotemporal lobe dementia—behavioral variant—with parkinsonism. Even when the density of presynaptic dopaminergic uptakers in the DaTSPECT was normal, she was put on Levodopa without significative clinical changes.

PERSPECTIVE

Clinical Observations

Parkinsonism is a well-known manifestation of FTLD (28). To date, presentations of FTLD with motor or movement disorders include (1) frontal lobe degeneration (FTD) with motor neuron disease (FTD-MND), (2) corticobasal degeneration (CBD), and (3) progressive supranuclear palsy (PSP). CBS and PSP usually show parkinsonism. However, parkinsonism has also been reported as a relative finding in other subtypes of FTD apart from CBS and PSP. Indeed, parkinsonism is found in approximately 20–30% of patients with FTLD. Furthermore, parkinsonism can be seen in all FTLD subtypes, and it can even be an outstanding feature in some cases. Therefore, there is a need to investigate parkinsonism in FTLD to obtain a better understanding of the disease.

Regarding the clinical characteristics, features of parkinsonism in FTLD are variable. The classical akinetic-rigid syndrome

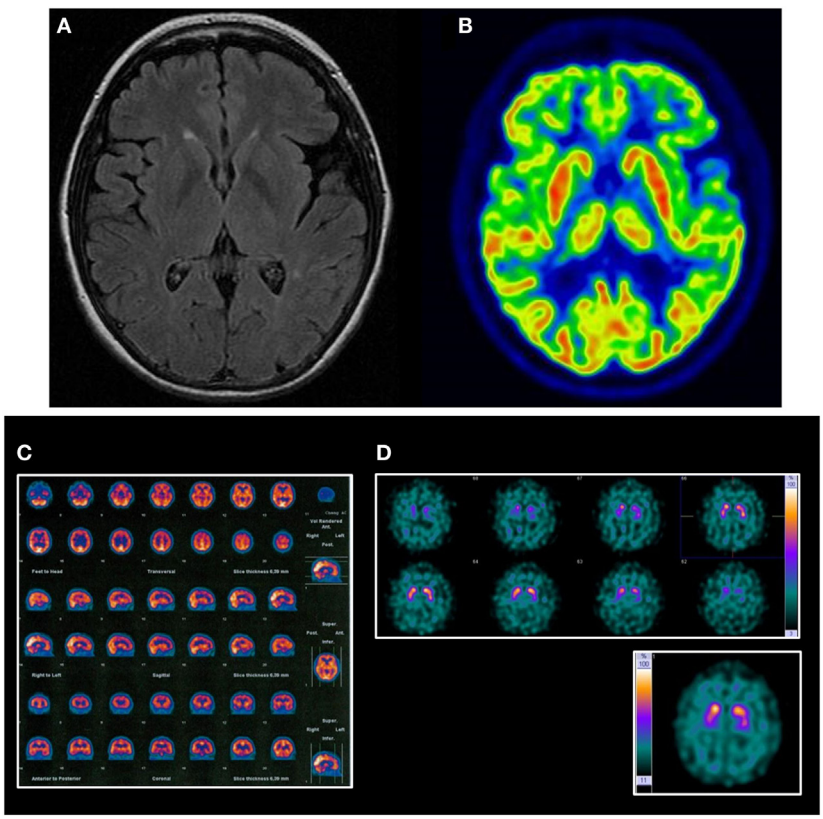

FIGURE 1 | Neuroimaging findings in the reported case. **(A)** Follow-up MRI mild diffuse atrophy with more intense atrophy on the left frontal and temporal lobes and some small vessel lesions affecting periventricular and semioval white matter. **(B)** FDG-PET shows mild-to-severe hypometabolism on the left frontotemporal lobe junction and mild hypometabolism on the left fronto-basal, left anterior cingulum regions. **(C)** HMPAO SPECT shows bilateral but asymmetric hypoperfusion (more on the left side) on frontotemporal lobes. **(D)** Dopamine transporter-single, photon emission computed tomography was informed of the normal density ofpresynaptic dopaminergic uptakers. Some irregularities in the morphology of basal ganglia and increase of cortical uptake can be noted.

usually characterizes it. Other cases show atypical parkinsonism resembling PSP or CBD. Although rare, parkinsonism in FTLD may coexist with MND. Parkinsonism in FTLD is usually levodopa unresponsive, but there have been cases where a temporary benefit has been reported. The lack of response to levodopa reinforces the idea of a pathogenic mechanism different to PD.

Genetics

A systematic review aimed at the characterization of movement disorder phenomenology in genetically proven familial FTLD, showed that at any point during the disease, parkinsonism was the most common movement syndrome. It was reported in 79.8% of cases, followed by PSP and CBD syndromes in 12.2 and 10.7% of cases, respectively (28). The amyotrophic lateral sclerosis/parkinsonism dementia complex of Guam was probably the first known association of parkinsonism with dementia of frontotemporal features (29). Parkinsonism was frequently observed in familial FTLD, more specifically in FTLD with parkinsonism linked to chromosome 17q (FTDP-17) (30). Parkinsonism in familial FTLD was first described in families with mutations in the microtubule-associated protein tau (MAPT) and progranulin (PRGN) genes. Since then, mutations in several other genes have been identified for FTLD with parkinsonism, including chromatin modifying protein 2B, chromosome 9 open reading frame 72 (C9ORF72), fused in sarcoma, valosin-containing protein, and transactive DNA-binding protein (TARDBP) (31, 32). Mutations in seven genes were robustly associated with autosomal dominant (SNCA, LRRK2, EIF4G1, VPS35) or recessive (parkin/PARK2, PINK1, DJ1/PARK7) PD or parkinsonism (33, 34). Changes in a long list of additional genes have also been suggested as causes for parkinsonism or PD, including genes for hereditary ataxias (ATXN2, ATXN3, FMR1), frontotemporal dementia (C9ORF72, GRN, MAPT, TARDBP), DYT5 (GCH1, TH, SPR), and others (ATP13A2, CSF1R, DNAJC6, FBXO, GIGYF2, HTRA2, PLA2G6, POLG, SPG11, UCHL1) (34–37).

Neuropsychology and DaTSPECT Imaging in PD and FTLD

Cognitive deficits could be identified in around a third of patients, even in the early untreated stages of PD (38–41). This cognitive dysfunction may have been related, in part, to reduced dopamine levels. While there were some inconsistent findings in the literature (42), fMRI studies with patients on and off levodopa indicated that higher levels of dopamine were associated with better cognitive performances on tasks of working memory and response accuracy (43). In addition, recent fMRI and DaTSPECT research have demonstrated a positive correlation between nigrostriatal dopaminergic function and performance on tests of executive functioning and memory (44). However, dopamine deficiency may not explain all of the cognitive deficits in PD, as some degree of cognitive impairment was common in patients diagnosed with related movement disorders such as dystonia (45) and essential tremor (46), conditions that were not associated with dopaminergic deficiency on imaging (13, 27, 36). These patients usually had an abnormal DaTSPECT as the damage in frontostriatal neural circuitry occurred down (basal ganglia) to up (frontal cortex) (47).

It is a well-known fact that cognitive impairment in early PD and other synucleinopathies (Parkinson-plus syndromes) was accompanied by reductions in activity in frontostriatal neural circuitry (48, 49). There were five fronto-subcortical circuits linking different regions of the frontal cortex and subcortical nuclei, involving several neurotransmitters. Nigrostriatal dopaminergic neurons played an important role in some of these circuits, but gabaergic, glutamatergic, and cholinergic neurons were also extensively represented. Therefore, neurodegenerative disorders affecting neurons outside the nigrostriatal dopaminergic circuits were not be detected by DaTSPECTs and would have still been able to produce extrapyramidal symptoms.

When dementia preceded PD, international consensus recommended a diagnosis of these patients with Dementia with Lewy bodies (DLB). Nevertheless, most patients with DLB and extrapyramidal signs had abnormal DaTSPECTs. In fact, autopsy studies provided class I evidence of 123I-FP-CIT dopaminergic neuroimaging accurately identifying patients with DLB (50), although this could be normal in those without extrapyramidal signs.

What is most relevant to the hypothesis presented in this paper is the fact that many SWEDD patients exhibited cognitive dysfunction. Studies with data from the Parkinson's Progression Marker Initiative (PPMI) database, showed that SWEDD patients performed significantly worse in semantic fluency and processing speed when compared with healthy controls (51). Montreal Cognitive Assessment test (MoCA) scores showed that about one-third of the PPMI participants were clinically diagnosed with SWEDD, experienced a statistically significant cognitive decline over the relatively brief period of 2 years, and a higher proportion of participants with SWEDD than DaTSPECT-confirmed PD had cognitive decline. The participants in the SWEDD group were more than twice as likely (relative risk = 2.07) than those in the dopamine-deficient group to fall below the MoCA cognitive impairment cutoff. Thus, recently diagnosed patients with SWEDD may have been an even more significant risk for cognitive decline than patients with DaTSPECT-confirmed early-stage PD (52, 53). Neuropsychological performances between the SWEDD and the PD resulted similar when comparing patients in all stages (54). Altogether, these results suggested that SWEDD should not be considered a benign finding, as a high proportion of patients could be expected to suffer a cognitive decline in the close future.

Some studies have shown decreased uptake of DaT in bilateral putamina in FTLD with a negative correlation between the uptake ratio and parkinsonian motor status (55). Another study by Morgan and colleagues found that around a third of the FTLD cases may have presented abnormal scan and a significant reduction in uptake in the putamen and the caudate (56). Therefore, patients with FTLD may have had a positive or a negative DaTSPECT. This result probably depends on the neuropathology and clinical subtype of FTLD.

For instance, a study with patients with non-fluent/agrammatic variant of primary progressive aphasia (nfvPPA) and a logopenic variant of PPA (lvPPA) without clinical parkinsonism

at baseline, showed reduced striatal tracer uptake in nfvPPA patients prior to clinical parkinsonism, especially for those nfvPPA without AD biomarkers, suggesting subclinical nigrostriatal degeneration (57). All lvPPA patients had normal DaTSPECT. Patients with nfvPPA presented abnormal DaTSPECT, especially in the left hemisphere compared with controls. DaTSPECT in nfvPPA patients with normal progranulin and negative CSF AD biomarkers was also significantly reduced compared with lvPPA patients with positive CSF AD biomarkers, suggesting nigrostriatal degeneration does not usually appear in the context of AD pathology resent, but it can appear in the context of FTD pathologies. During follow-up, seven nfvPPA/bio-patients developed parkinsonism, six of them with baseline reduced 123I-FP-CIT uptake (57).

FINAL REMARKS

The literature shows many clinical, genetic, and neuropsychological studies linking parkinsonism and FTLD. Also, a good number of articles provide evidence of the distinct characteristics between PD and SWEDD, and on the cognitive impairment present in many SWEDD cases. Finally, DaTSPECT studies in FTLD found both positive and negative results.

We report the case of a patient diagnosed with a behavioral variant of frontotemporal lobe dementia who also has parkinsonism that was not attributable to vascular lesions, antidopaminergic drugs, or any other etiology. DaTSPECT was informed as normal, and therefore it might be considered SWEDD.

On this basis, we reinforce the observation that parkinsonism without evidence of nigrostriatal dopaminergic deficit can be present in FTLD patients. Even when most of them will also present cognitive impairment, parkinsonism may be the main reason for consultation, and a normal DaTSCAN can be found during the diagnostic process. Therefore, FTLD may account for a percentage of patients with SWEDD. This origin might contribute to the observation that SWEDD patients exhibit a higher risk of cognitive impairment, worse mood, and better olfaction than PD patients (19, 51). Other causes of SWEDD have been described, particularly dystonic tremor. Thus, SWEDD should be understood as a heterogeneous group of patients in which some cases (probably the most tremoric ones) may have dystonic tremor, and some others may have FTLD (probably those with outstanding frontal cognitive dysfunction).

Careful clinical assessment remains the cornerstone of the study of patients with FTLD with parkinsonism. However, rationale support of genetic studies, neuropsychological evaluation, and neuroimaging, including structural and functional techniques, will provide a more detailed and comprehensive picture.

ETHICS STATEMENT

Written informed consent was obtained from the patient for the publication of this case report.

AUTHOR CONTRIBUTIONS

All authors contributed equally.

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Therapeutic Interventions for Vascular Parkinsonism: A Systematic Review and Meta-analysis

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Background: Vascular parkinsonism (VP) is defined as the presence of parkinsonian syndrome, evidence of cerebrovascular disease, and an established relationship between the two disorders. However, the diagnosis of VP is problematic, particularly for the clinician confronted with moving from diagnosis to treatment. Given the different criteria used in the diagnosis of VP, the effectiveness of available therapeutic interventions for this disease are currently unknown.

Methods: To assess the clinical response of all published therapeutic interventions for VP that have been reported in the literature, we conducted a systematic review looking for VP subjects treated with any therapeutic intervention. To clarify the prevalence of responsiveness to levodopa among VP subjects, we conducted a meta-analysis of 17 observational studies retrieved with the search criteria of our review. Also, four studies were included in a second analysis to explore if nigrostriatal lesion affected the prevalence of levodopa response in VP subjects. Relevant articles were identified from MEDLINE, Scopus, and Web of Science published until June 2017.

Results: 436 non-duplicate citations were identified for screening, 107 articles were assessed for eligibility, and only 23 observational studies were included in this review. No randomized clinical trials were found. Four different therapies were found in the literature; among them, levodopa was the only one repetitively reported. The calculated event rate of levodopa response in VP subjects was of 0.304 [95% confidence interval (CI) of 0.230–0.388]. The overall odds ratio for good response to levodopa in VP with lesion in the nigrostriatal pathway vs. no lesion in the nigrostriatal pathway was 15.15 (95% CI: 5.2–44.17).

Conclusion: Despite the lack of randomized controlled trials, results of this systematic review and meta-analysis show that VP subjects, as operationally defined here, have a low response rate to levodopa; nigrostriatal lesion could be used as a proxy predictor of levodopa response in VP subjects. Other therapies seem to be co-adjuvant. Randomized controlled trials with a clear definition of VP are necessary to be able to assign positive or negative predictive values to available treatments and to recommend any of the therapeutic interventions for these subjects.

Keywords: vascular parkinsonism, therapy, treatment, systematic review, meta-analysis

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INTRODUCTION

Vascular parkinsonism (VP) is defined as the presence of parkinsonian syndrome, unequivocal evidence of cerebrovascular disease, and an established relationship between the two disorders (1, 2). The predominantly reported clinical manifestation of VP is lower-body parkinsonism (mainly impaired gait with unstable posture, poor response to levodopa, difficulty maintaining balance and frequently, exhibition of freezing) (3). Imaging studies have been used to corroborate the diagnosis of VP, but given the prior probability of vascular disorders in the older population, these studies established correlation but not causation (4). The criteria of Zijlmans et al. (1) (**Table 1**) moves toward establishing causation by imposing time constraints between the diagnosis of parkinsonism and imaging, although it is difficult to precisely ascertain the onset of parkinsonism. Ever since the concept of VP was first introduced by Critchley in 1929 (5), its existence has been subject to debate due to the lack of consensus regarding its diagnostic criteria. The broad spectrum of reported cases has been variably referred to in the literature as arteriosclerotic parkinsonism, arteriosclerotic pseudo-parkinsonism, pseudo-VP, vascular pseudo-parkinsonism, and lower-body parkinsonism, even when the physiopathology differs from that of VP (1, 2, 6).

Although the definition of this clinical syndrome is controversial, according to literature, it accounts for approximately 4.4–12% of all patients with parkinsonism (7, 8). The incidence of VP is expected to rise due to an increasingly aging population and the heavier burden of vascular risk factors this entails (9). However, except for controlling vascular risk factors, there is currently no first-line treatment for patients with VP (3).

Levodopa is the most effective treatment for Parkinson's disease (PD), and in spite of the clinical similarities between VP and PD, VP has been widely characterized as a parkinsonism that is not responsive to levodopa (3). This statement was challenged by Zijlmans et al. in 2004 (10); in a retrospective clinicopathological study, VP subjects with vascular lesions in or near the nigrostriatal pathway showed good response to levodopa regardless of their parkinsonism onset type (acute or insidious) or their dominant clinical features (10). Since then, no other studies have aimed specifically to test levodopa response in patients with a diagnosis of VP.

An anecdotal review from 2007 reported the levodopa responsiveness in different kinds of parkinsonism. It stated that VP was responsive to levodopa in 20–40% of patients (11); however, this review was not focused exclusively on VP, and its criteria for inclusion had a VP definition that was too lax and, therefore, possibly containing a bias toward misdiagnosed VP. Recent studies have used more structured criteria to confirm diagnosis of VP (**Tables 1** and **2**).

A recent cohort study on parkinsonian subjects revealed that PD subjects present a better prognosis compared to subjects with VP, who have a greater rate of institutionalization and a mortality ratio of 3 years (12). Their findings emphasize the important clinical differences and prognoses between parkinsonisms. Good levodopa response has been widely used as a prospective criteria for PD diagnosis (13), and lack of responsiveness to levodopa in a patient with parkinsonian syndrome is frequently used to

TABLE 1 | Zijlmans' vascular parkinsonism criteria.

Zijlmans' diagnostic criteria[1]
Step 1. Parkinsonian syndrome
• Bradykinesia
At least 1 of the following:
• Rest tremor • Muscular rigidity • Postural instability not caused by primary visual, vestibular, cerebellar, or proprioceptive dysfunction
Step 2. Cerebrovascular disease
• Evidence of relevant cerebrovascular disease by brain imaging: CT or MRI.
AND/OR
• Presence of focal signs or symptoms that are consistent with stroke.
Step 3. An established relationship between the parkinsonism and the cerebrovascular disease
• Acute VP: an acute or delayed progressive onset with infarcts in or near areas that can increase the basal ganglia motor output (GPe or substantia nigra pars compacta) or decrease the thalamocortical drive directly (VL of the thalamus, large frontal lobe infarct). The parkinsonism at onset consists of a contralateral bradykinetic rigid syndrome or shuffling gait, within 1 year after a stroke
OR
• Insidious VP: an insidious onset of parkinsonism with extensive subcortical white matter lesions, bilateral symptoms at onset, and the presence of early shuffling gait or early cognitive dysfunction
Step 4 Exclusion criteria for VP
• History of repeated head injury • Definite encephalitis • Neuroleptic treatment at onset of symptoms • Presence of cerebral tumor or communicating hydrocephalus on CT or MRI scan • Other alternative explanation for parkinsonism

CT, computed tomography; GPe, globus palidus extern; MRI, magnetic resonance imaging; VP, vascular parkinsonism; VL, ventrolateral.

TABLE 2 | Winikates' vascular parkinsonism criteria.

Winikates' diagnostic criteria (16)
Step 1. Parkinsonian syndrome Presence of at least 2 of the 4 cardinal signs of parkinsonism:
• Tremor at rest • Bradykinesia • Rigidity • Loss of postural reflexes.
Step 2. Vascular score of 2 points or more
• 2 points: pathologically or angiographically proven diffuse vascular disease. • 1 point: onset of parkinsonism within 1 month of clinical stroke. • 1 point: history of 2 or more strokes. • 1 point: neuroimaging evidence of vascular disease in 2 or more vascular territories. • 1 point: history of 2 or more risk factors for stroke.
Risk factors for stroke: hypertension, smoking, diabetes mellitus, hyperlipidemia, presence of heart disease associated with stroke (coronary artery disease, atrial fibrillation, congestive heart failure, valvular heart disease, mitral valve prolapse, other arrhythmias), family history of stroke, history of gout, and peripheral vascular disease

pinpoint VP, even though responsiveness to levodopa is unclear in VP subjects.

This review aims to examine the clinical effects of the current pharmacological and non-pharmacological therapies for VP and to answer the following questions: (1) does available literature affirm the assertion that VP subjects are non-responders to levodopa? (2) Does nigrostriatal lesion modify the levodopa response rate in VP subjects? (3) How does VP subject's response rate to levodopa therapy differ from PD subjects?

To answer these questions, we conducted a systematic review of available literature, looking for original articles that assessed response to different therapeutics in VP subjects. Also, we conducted a meta-analysis on the prevalence of response to levodopa therapy of VP subjects. Then, we conducted a second meta-analysis to assess if lesion of the nigrostriatal pathway affects levodopa response in VP subjects. Finally, VP subject's levodopa response rate was compared to that of PD subjects to assess the validity of this parameter for differential diagnosis.

METHODS

Search Strategy

In this study, we conducted a systematic review of the literature using the Preferred Reporting Items for Systematic reviews and Meta-Analyses model (14). Relevant articles were identified from MEDLINE, Scopus, and Web of Science published until June 2017. No registered clinical trials were identified from http://clinicaltrials.gov or http://clinicaltrialsregister.eu. Our search was aimed to identify studies that reported the clinical response to different kinds of therapeutic interventions in adult subjects with VP diagnosis. As historically VP has been poorly defined, we included only those studies that clearly and systematically defined VP. Given the difficulties of establishing VP diagnosis, all results and conclusions must take into account these operational definitions (Tables 1 and 2). To avoid possible bias, studies that used levodopa response as part of the definition of VP were not included. We used the following terms and Mesh terms (medical subject headings): PD, secondary; parkinsonian disorders; vascular; blood vessels; therapeutics; vitamin D; ergocalciferols; levodopa; amantadine; aripiprazole; transcranial magnetic stimulation. Full details on the search algorithm can be found in the Supplemental data. Further analysis of the references of each article was carried out to find articles that could have been excluded by the search algorithm. Only articles published in English were considered. Also, using the information retrieved by the search criteria of this systematic review, we conducted a meta-analysis focused on the prevalence of response VP subjects have to levodopa therapy. We followed the MOOSE guidelines for conducting meta-analyses of observational studies (15). Results of the search strategy are summarized in **Figure 1**.

Inclusion Criteria

Studies meeting the following criteria were included in the systematic review: (1) the study's design had to be experimental or observational (clinical trials, clinicopathological studies, cohort studies, cross-sectional observational studies, case series, and case–control studies; case reports of less than five subjects were not included in the analysis due to poor external validity of this kind of studies), (2) studies had to be explicitly focused on VP, (3) they had to report pharmacological or non-pharmacological intervention, along with the clinical response of the subjects to treatment, (4) articles had to contain a diagnosis of VP with

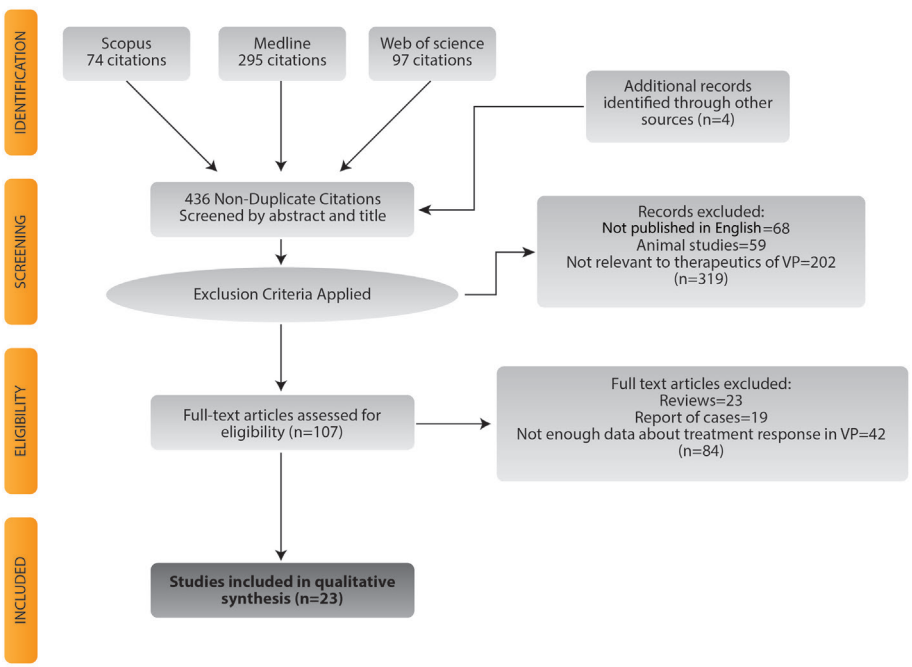

FIGURE 1 | Flowchart for study selection.

an explicit criteria, whether known criteria [e.g., Zijlmans' (**Table 1**) (1) or Winikates' (16) (**Table 2**) criteria] or any other specified criteria (5) studies could not have levodopa response as part of the VP definition.

For the meta-analysis, studies were included if they met the above-mentioned criteria, but with the following substitution: (3) levodopa treatment had to be reported, along with the clinical response of the subjects [if the clinical response was measured as Unified Parkinson's Disease Rating Scale (UPDRS) (%) reduction, then reduction had to be reported for every patient and cutoff points established to determine whether the subject was responsive or not to levodopa therapy].

Study Selection

Two of the authors (Adán Miguel-Puga and Gabriel Villafuerte) carried out the eligibility assessment of the studies independently (15). Any discrepancy was adjudicated by consensus with a third author (Oscar Arias-Carrión). The initial evaluation of the references consisted of an analysis of the title and abstract for each screened reference. Full texts of relevant articles were then retrieved to complete the examination and eligibility process. **Figure 1** shows the flowchart for study selection.

Data Extraction

Extraction of data was carried out first by one author (Adán Miguel-Puga) and then checked by another one (Gabriel Villafuerte). The information extracted for each study included: name of first author, year of publication, continent where the study was performed, type of study design, characteristics of the study population, the diagnosis criteria for VP, the number of VP subjects (and PD if applicable) included in each study, the existence of confirmed vascular lesions and the clinical response to the intervention.

Quality Assessment

The quality assessment was performed by two authors independently (Adán Miguel-Puga, Gabriel Villafuerte) using the STROBE checklist (17) for observational studies, which is a 22-point checklist. Any discrepancy was adjudicated by consensus with a third author (Oscar Arias-Carrión). The quality of the articles was evaluated according to the checklist. The articles were scored according to the following criteria: a point was given for every item from the checklist that was included in the study; if the item was not considered or it was impossible to determine whether it was considered or not, no point was given. A summary of the number of points obtained by each study can be found in **Table 3**.

Statistical Analysis

The statistical analysis was calculated using Comprehensive Meta-Analysis software V3. Forest plots were constructed with Graphpad Prism. Three different analyses were conducted: one to investigate the prevalence of levodopa response in VP subjects, one to assess whether the presence of nigrostriatal lesion modified the levodopa response rate in VP subjects, and one to compare the prevalence of response among VP subjects and PD subjects. To calculate the pooled effect size, both fixed and random effect models were implemented; as high heterogeneity was expected, only random effect results are reported. Fixed effect results can be consulted in the Supplementary Material. Heterogeneity was estimated using I^2 and Tau^2; I^2 was computed using the fixed effect weights. Studies with I^2 values from 0 to 25% were considered as having low heterogeneity, studies with values ranging from 25 to 50% were considered as having moderate heterogeneity and studies showing more than 50% were assessed as presenting high heterogeneity. To assess heterogeneity, we conducted diverse subgroup analyses to identify the origin of the heterogeneity: low vs. high-quality analysis, analysis by type of publication and analysis by continent of publication. Given the characteristics of the data extracted, no meta-regression could be done. For low- vs. high-quality analysis, we used the STROBE checklist score to divide the studies into two subjective groups (studies with 15 or less were considered as low quality, and more than 15 were considered as high quality; **Table 3**).

Because we found three different criteria that were being used for VP diagnosis, we made a subgroup analysis to investigate if the diagnostic criteria influenced the results of the overall pooled effect and heterogeneity; subgroups were combined with a random effects model. We also carried out a sensitivity analysis to estimate the influence of each study on the overall effect size; this analysis was realized by omitting one study at a time and then recalculating the effect size. Egger's and Begg's tests were conducted to detect possible publication bias; also, funnel plots were constructed for each of these analyses (**Figure 2**).

RESULTS

What are the Clinical Effects of the Current Pharmacological and Non-Pharmacological Therapies for VP?

A total of 23 studies were included and analyzed in a qualitative revision. **Figure 1** summarizes the study identification and selection process. Four different therapies were identified: 1 study investigated vitamin D therapy (24), 1 study assessed repetitive transcranial magnetic stimulation (rTMS) therapy (28), 1 study was focused on lumbar puncture as therapy (32) and 20 studies reported levodopa therapy (10, 16, 18–23, 25–27, 29–31, 33–38). Clinical trials were not found for any therapeutic. Pilot studies were retrieved for rTMS (28) and lumbar puncture therapy (32). A case–control study was found in the vitamin D study (24). For levodopa, we obtained 14 cross-sectional studies (16, 18–23, 29–31, 33, 35, 37, 38), 2 case–control studies (25, 27), 2 cohort studies (26, 34), and 2 clinicopathological studies (10, 36). All studies are summarized in **Table 4**. Specific characteristics of every study are depicted on Tables S1–S3 in Supplementary Material.

As the main bias in the research of VP is the criteria used to define a VP case, we divided the studies according to the diagnosis criteria utilized. The majority of the studies used either Zijlmans et al.'s criteria (1) (Table S1 in Supplementary Material) or Winikates' criteria (16) (Table S2 in Supplementary Material). Zijlmans' criteria was used for the diagnosis of VP in the vitamin

TABLE 3 | STROBE checklist evaluation.

STROBE STATEMENT CHECKLIST

Studies	Vascular parkinsonism criteria	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
Vale et al. 2015	Zijlmans'																							16
Lee et al. 2015	Zijlmans'																							11
Gago et al. 2015	Zijlmans'																							15
Navarro Otano et al. 2014	Zijlmans'																							19
Jang et al. 2014	Zijlmans'																							15
Vale et al. 2013	Zijlmans'																							9
Sato et al. 2013	Zijlmans'																							16
Benitez Rivero et al. 2013	Zijlmans'																							20
Antonini et al. 2012	Zijlmans'																							18
Zijlmans et al. 2007	Zijlmans'																							10
Zijlmans et al. 2004	Zijlmans'																							9
Yip et al. 2013	Winikates'																							16
Kim et al. 2006	Winikates'																							11
Katzenschlanger et al 2004	Winikates'																							8
Lorberboym et al. 2004	Winikates'																							8
Ondo et al. 2002	Winikates'																							12
Huang et al. 2002	Winikates'																							9
Winikates et al. 1999	Winikates'																							17
Rampello et al. 2005	Other																							8
Demirkiran et al. 2001	Other																							7
Yamanouchi et al. 1997	Other																							11
Zijlmans et al. 1996	Other																							8
Zijlmans et al. 1995	Other																							11

COLOR CODE	
	Item reported
	Item not reported
	High quality studies
	Low quality studies

D study (24) and in 10 of the levodopa studies (10, 18–23, 25–27) (Table S1 in Supplementary Material). Meanwhile, Winikates' criteria were used for the diagnosis of VP in the rTMS and lumbar puncture studies (28, 32) and in five of the levodopa studies (16, 29–31, 33) (Table S2 in Supplementary Material). The studies that used neither Zijlmans' nor Winikates' criteria but clearly specified how the VP diagnosis was made are summarized in Table S3 in Supplementary Material, together with the criteria that were utilized in these studies (34–38).

The study on vitamin D therapy was a case–control study that was carried out on a Japanese population. It included a total of 178 subjects, 90 of them with a VP diagnosis according to Zijlmans' criteria (1). The study evaluated the effectiveness of vitamin D therapy for prevention of falls and hip fractures. After 2 years of treatment with 1,200 UI/day of ergocalciferol, it was reported that VP subjects had 18% fewer falls compared with PD subjects ($p < 0.001$), no change in parkinsonian symptoms were observed (24).

The rTMS pilot study was carried out in Singapore; it included a total of five VP subjects. Winikates' criteria was used for the VP diagnosis (16). The main outcomes reported were changes in the timed 10-m walk test and the score given by the UPDRS part 3. The rTMS protocol used was 20 trains of 10 s each, with 5 Hz at 80% of active motor threshold. The study showed reduced scores for the UPDRS part 3 at week 2 ($p = 0.004$), 4 ($p = 0.022$), and 6 ($p = 0.046$) and significant improvement in the timed 10-m walk test at week 2 ($p = 0.059$) and 4 ($p = 0.026$) but not at week 6 as compared to baseline (28).

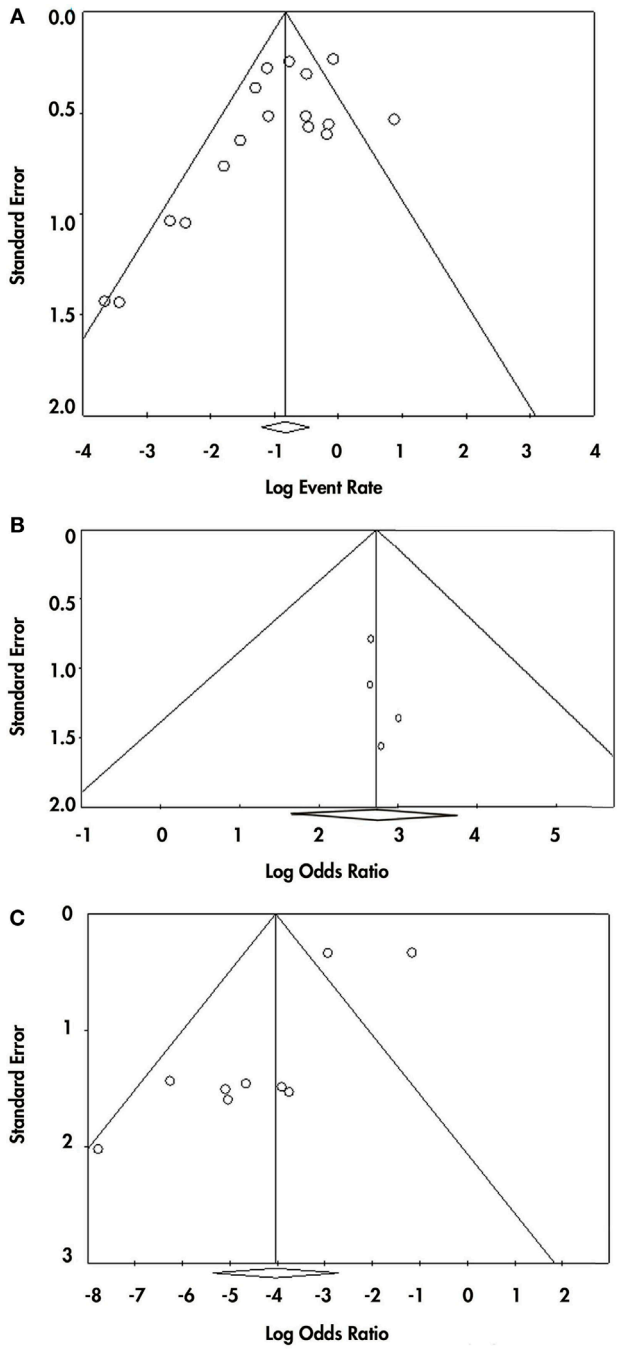

FIGURE 2 | Publication bias for the three analysis made. Panel **(A)** shows the funnel plot from the event-rate analysis. Panel **(B)** shows the funnel plot from the prevalence of responsiveness in vascular parkinsonism (VP) subjects with nigrostriatal lesion vs. VP subjects without nigrostriatal lesion. Panel **(C)** shows the funnel plot from the prevalence of responsiveness in VP vs. Parkinson disease.

Lumbar puncture therapy was carried out in a pilot study that included 40 American subjects with a VP diagnosis by Winikates' criteria (16). 35–40 cc of cerebrospinal fluid (CSF) was drained from each subject. Out of 40 subjects, 15 showed a good subjective improvement after therapy, while the rest had mild or no improvement. The mean duration of the therapeutic response was 2.4 ± 1.2 months (32).

Finally, treatment with levodopa was evaluated in 14 cross-sectional studies (16, 18–23, 29–31, 33, 35, 37, 38), 2 case–control studies (25, 27), 2 cohort studies (26, 34), and 2 clinicopathological studies (10, 36). Except for one clinicopathological study (10), no other study was specifically aimed at testing the levodopa response of VP subjects. For the studies in which Zijlmans' criteria was used (10, 19, 21, 22, 25–27), a total of 93 VP subjects showed a favorable response to levodopa, while 155 VP subjects showed no response (a prevalence of responsiveness in 37.5% of the subjects). On the other hand, a good response was reported in 323 PD subjects and no response in just 23 of them (90% of the subjects were responsive). In the five studies (16, 29–31, 33) that used Winikates' criteria (16), a total of 30 subjects responded well to levodopa (a response rate of 22.05% of the subjects), while 106 subjects were non-responsive. The 5 studies with no specific criteria (34–38) showed a similar rate of response as the studies using the Winikates' criteria: a total of 78 subjects had no response, while 25 subjects (24.27%) responded favorably to levodopa.

Four studies (18, 20, 23, 27) measured the (%) of improvement on the UPDRS part 3 of VP subjects after levodopa therapy; respective sample sizes were: 13, 17, 5, and 15 subjects; reduction of motor symptoms ranged from 5.8 to 22.25%. Two studies (18, 20) compared the UPDRS reduction to that of PD patients; VP subjects showed a reduction of 5.9–18.7% compared to 31.6–64.65% in PD subjects.

Three studies with a sample size of 20, 76, and 42 VP subjects reported that nigrostriatal dopaminergic denervation [evidenced by an abnormal fluoropropyl-2b-carbomethoxy-3b-(4-iodophenyl)-tropane single photon emission computed tomography (FP-CIT SPECT)] may predict a favorable response to levodopa in VP subjects (19, 26, 31).

Does Available Literature Affirm the Assertion that VP Subjects are Non-Responders to Levodopa?

To determine the prevalence of favorable response to levodopa therapy among VP subjects, a meta-analysis of the data was conducted.

A total of 17 studies were included in the meta-analysis. For this analysis, the following studies are summarized; 13 cross-sectional studies (10, 16, 19, 21, 22, 29–31, 33, 35–38), 2 case–control studies (25, 27), 2 cohort studies (26, 34) (clinicopathological studies were considered as cross-sectional studies). Of these studies, 2 studies were conducted in America (16, 33), 10 in Europe (10, 21, 25–27, 30, 34, 35, 37, 38), and 5 in Asia (19, 22, 29, 31, 36). All studies included both male and female subjects, but the response to levodopa was not divided by sex, so this variable could not be included in the meta-analysis. According to the year of publication, 13 studies were published after the year 2000 (10, 19, 21, 22, 25–27, 29–31, 33–35), while the remaining 4 studies were published in the year 2000 or earlier (16, 36–38). The estimated quality of all included studies was in the range of 7–20 points on the

TABLE 4 | Summary of included studies.

	Studies	Continent	Type of study	Treatment	Focused on treatment	Response to treatment	Comments
Zijlmans' Criteria	Vale et al. (18)	America	Cross-sectional	Levodopa	NO	Poor	
	Lee et al. (19)	Asia	Cross-sectional	Levodopa	NO	Poor	VP subjects with nigrostriatal dopaminergic denervation had better response to levodopa
	Gago et al. (20)	Europe	Cross-sectional	Levodopa	NO	Poor	
	Navarro-Otano et al. (21)	Europe	Cross-sectional	Levodopa	NO	Poor	
	Jang et al. (22)	Asia	Cross-sectional	Levodopa	NO	Poor	
	Vale et al. (23)	America	Cross-sectional	Levodopa	NO	Poor	Poor reduction of motor UPDRS score with levodopa
	Sato et al. (24)	Asia	Case-control	Vitamin D	YES	Good	Decreased risk of falls with vitamin D
	Benitez-Rivero et al. (25)	Europe	Case-control	Levodopa	NO	Poor	
	Antonini et al. (26)	Europe	Cohort	Levodopa	NO	Poor	VP subjects with normal FP-CIT SPECT and/or LS in basal ganglia are unlikely to respond to levodopa
	Zijlmans et al. (27)	Europe	Case-control	Levodopa	NO	Poor	Poor reduction of motor UPDRS score with levodopa
	Zijlmans et al. (1, 10)	Europe	Clinicopathological	Levodopa	YES	Good	Good response to levodopa was related to lesions in or near the nigrostriatal pathway
Winikates' Criteria	Yip et al. (28)	Asia	Pilot study	rTMS	YES	Good	VP dysfunction could be improved with rTMS
	Kim et al. (29)	Asia	Cross-sectional	Levodopa	NO	Poor	
	Katzenschlager et al. (30)	Europe	Cross-sectional	Levodopa	NO	Mixed	
	Lorberboym et al. (31)	Asia	Cross-sectional	Levodopa	NO	Poor	Normal ^{123}I-FP-CIT FP-CIT SPECT may predict a poor response to levodopa
	Ondo et al. (32)	America	Pilot study	Lumbar puncture	YES	Mixed	Subjective improvement. Subjects responsive to lumbar puncture had better response to levodopa
	Huang et al. (33)	America	Cross-sectional	Levodopa	NO	Poor	
	Winikates and Jankovic (16)	America	Cross-sectional	Levodopa	NO	Poor	
Other Criteria	Rampello et al. (34)	Europe	Cohort	Levodopa	NO	Poor	
	Demirkiran et al. (35)	Europe	Cross-sectional	Levodopa	NO	Poor	
	Yamanouchi and Nagura (36)	Asia	Clinicopathological	Levodopa	NO	Poor	
	Zijlmans et al. (37)	Europe	Cross-sectional	Levodopa	NO	Poor	
	Zijlmans et al. (38)	Europe	Cross-sectional	Levodopa	NO	Poor	

^{123}I-FP-CIT SPECT, ^{123}I –labeled fluoropropyl-2b-carbomethoxy-3b-(4-iodophenyl)-tropane single photon emission computed tomography; LS, lacunar strokes; rTMS, repetitive transcranial magnetic stimulation; UPDRS, Unified Parkinson's Disease Rating Scale; VP, vascular parkinsonism (VP).

STROBE checklist (17). These ratings have been reported in **Table 3**.

The results of the event-rate meta-analysis of the prevalence of levodopa response in subjects with VP are reported in **Figure 3**. The levodopa response of a total of 487 VP subjects distributed in 17 studies was included in this analysis. The overall event rate found was of 0.304 [95% confidence intervals (CI) of 0.230–0.388]. With the subgroup analysis, we found that the event rate changed depending on the diagnostic criteria; however, CI 95% overlapped (Supplementary Material). Sensitivity analysis showed event rates from 0.287 (CI 95% 0.234–0.402) to 0.316 (CI 95% 0.241–0.400) (Supplementary Material). High heterogeneity was found (I^2 = 61.37% and Tau^2 = 5.65). The subgroup analysis showed that all the VP diagnosis criteria had heterogeneity (I^2 "Zijlmans'" = 60.5%, I^2 "Winikates'" = 33.153%, I^2 "Other" = 68.11%) and that quality of the studies did not influence heterogeneity (I^2 high-quality studies = 73.12% and I^2 low-quality studies = 60.06%) (Supplementary Material). Changes in heterogeneity distribution were found in the subgroup analysis for continent and type of study. Continent subgroup analysis showed that American studies had no heterogeneity (I^2 = 0%), while the heterogeneity concentrated in Asian and European studies was high (I^2 = 55.44% and I^2 = 58.43%, respectively) (Supplementary Material). Type of study subgroup analysis indicated that case and control studies and cohort studies had no heterogeneity (both with I^2 = 0%), while the heterogeneity was produced by the cross-sectional studies (I^2 = 60.87%) (Supplementary Material). Information of sensitivity and group subanalysis data are depicted on Tables S4–S8 in Supplementary Material.

Visual inspection of funnel plot showed asymmetry in the inferior part of the plot, so publication bias remains a possibility (Begg test p-value = 0.02 and Egger test p-value = 0.05) for this analysis (**Figure 2A**).

Does Nigrostriatal Lesion Modify the Levodopa Response Rate in VP Subjects?

For the second meta-analysis, we pooled the odds ratio (OR) of the probability of responding to levodopa in VP subjects

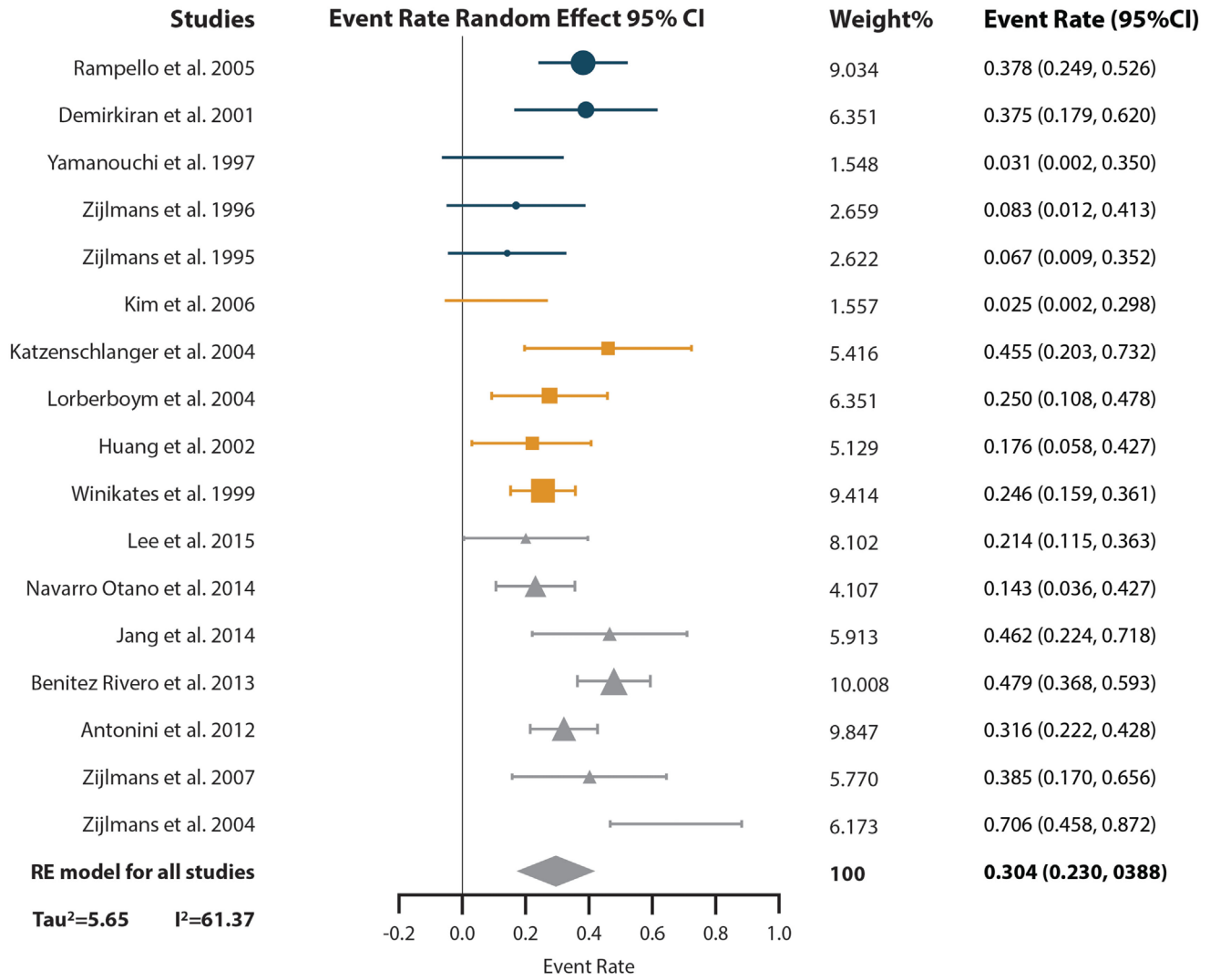

FIGURE 3 | Pooled random effect event rate and 95% confidence interval (CI) for the prevalence of levodopa response in vascular parkinsonism subjects. Circles represent studies with the "Other" diagnostic criteria, squares represent studies with the "Winikates'" diagnostic criteria, and triangles represent "Zijlmans'" diagnostic criteria. Size of the geometrical figures is proportional to their respective relative weight. RE, random effect.

with nigrostriatal lesion compared with VP subjects without nigrostriatal lesion (**Figure 4**). A total of 155 VP subjects were included: 90 with nigrostriatal lesion and 65 without lesion. The subjects were distributed in four studies. The pooled OR showed that VP subjects with nigrostriatal lesion are much more likely to respond to levodopa (OR = 15.148, 95% CI 5.195–44.169). Minimal heterogeneity is inferred in this analysis as demonstrated by an $I^2 = 0\%$ and a $Tau^2 = 0$. Due to the lack of heterogeneity, fixed and random effect, pooled effect and relative weights were the same. No subgroup analysis was performed due to lack of heterogeneity. Sensitivity analysis did not change the tendency in the effect as it had an OR ranging from 14.36 (95% CI 4.46–46.19) to 16.14 (95% CI 3.66–71.16) (Supplementary Material). Funnel plot inspection did not demonstrate asymmetry and the Egger and Begg *p*-values were above 0.05 (**Figure 2B**).

How does VP Subject's Response Rate to Levodopa Therapy Differ from Parkinson's Disease Subjects?

To answer this question, we performed a third meta-analysis where we pooled the OR of the probability VP subjects have of responding to levodopa compared with the probability of PD subjects (**Figure 5**). The response of a total of 340 VP subjects and 734 PD subjects distributed in nine studies were included in this analysis. An overall OR of 0.018 (CI 95% 0.005–0.066) was found. A high heterogeneity measure was found ($I^2 = 80.27\%$ and $Tau^2 = 2.50$). The subgroup analysis by VP diagnosis criteria also evidenced different ORs and 95% CI, although the CIs overlapped (Supplementary Material). This same subgroup analysis by VP diagnosis criteria exhibited no heterogeneity in the "Other" subgroup ($I^2 = 0\%$), while great heterogeneity in the "Zijlmans'"

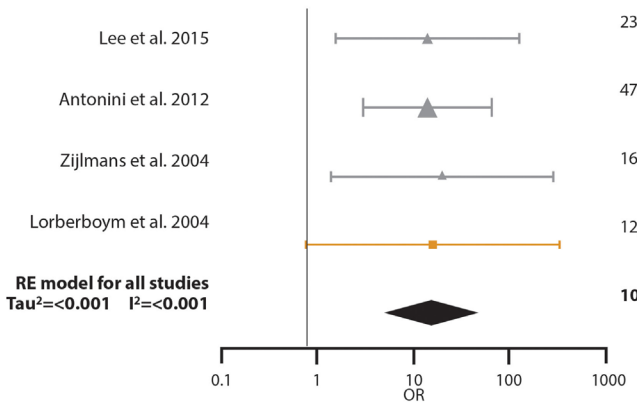

FIGURE 4 | Pooled odds ratio (OR) and 95% confidence interval (CI) for the probability of vascular parkinsonism (VP) subjects responsiveness to levodopa with nigrostriatal lesion compared with VP subjects without nigrostriatal lesion. Circles represent studies with the "Other" diagnostic criteria, squares represent studies with the "Winikates'" diagnostic criteria, and triangles represent "Ziljmans'" diagnostic criteria. Size of the geometrical figures is proportional to their respective relative weight. RE, random effect.

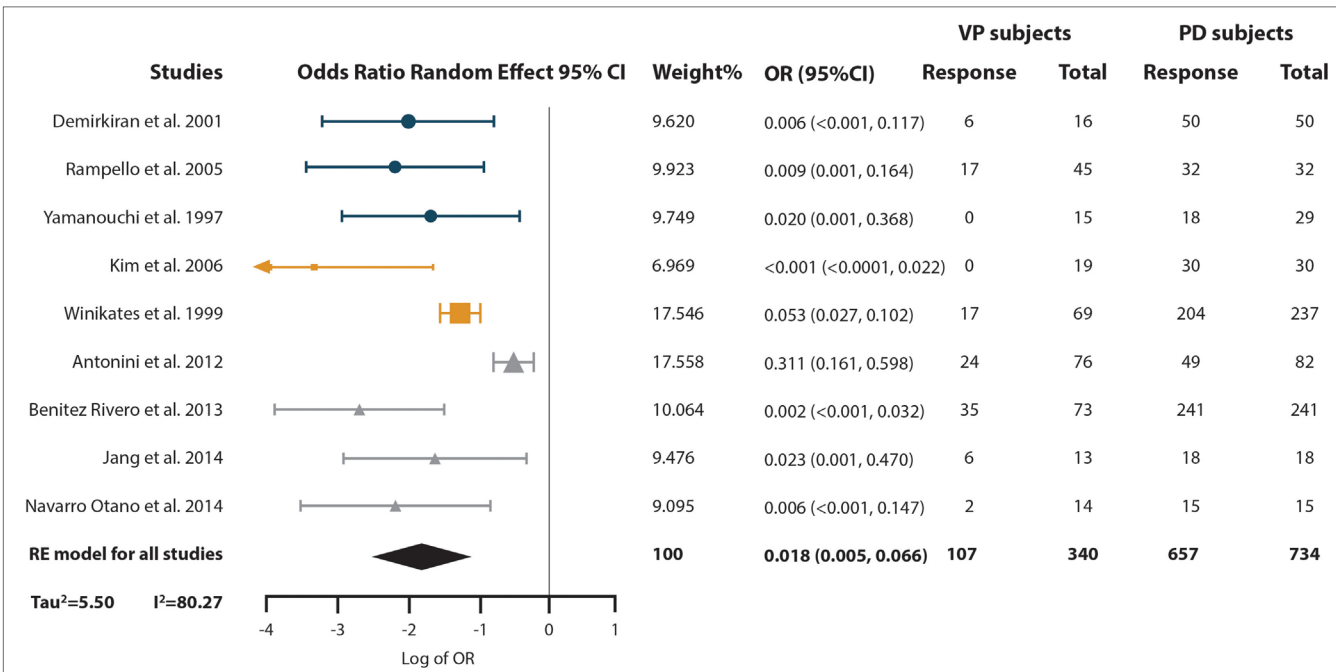

FIGURE 5 | Pooled odds ratio (OR) and 95% confidence interval (CI) for the probability of vascular parkinsonism (VP) subjects responsiveness to levodopa compared with the probability of PD subjects response to it. Circles represent studies with the "Other" diagnostic criteria, squares represent studies with the "Winikates'" diagnostic criteria, and triangles represent "Zijlmans'" diagnostic criteria. Size of the geometrical figures is proportional to their respective relative weight. RE, random effect.

and "Winikates'" subgroup was found (I^2 = 84.06 and 82.04%, respectively) (Supplementary Material). Surprisingly, subgroup analysis for quality showed null heterogeneity in the low-quality group (I^2 = 0%) whereas high heterogeneity was found in the high-quality group (I^2 = 88.25%) (Supplementary Material). Sensitivity analysis pointed out ORs ranging from 0.011 (95% CI 0.003–0.038) to 0.024 (95% CI 0.007–0.089) Supplementary Material (para unificarlo con todos). Funnel plot showed asymmetry, especially in the upper part of the graph and statistical analysis for publication bias showed evidence of this bias (Begg

test p-value = 0.67 and Egger test p-value = 0.01); however, results remain solid due to a "classic fail-safe N": the calculated number of studies missing needed to bring the p-value greater than 0.05 would be 315 (**Figure 2C**). Information of sensitivity and group subanalysis data are depicted on Tables S9–S14 in Supplementary Material.

DISCUSSION

Our systematic review of the available literature revealed that few studies had been done on potential therapeutics for VP. Also, the evidence retrieved of the proposed VP therapies comes from observational studies and not from prospective and controlled studies.

Subjects treated with rTMS showed clinical improvement as validated by the timed 10-m walk test and UPDRS part 3 (28). However, the study had no sham control and therefore no blindness; results could still be adjudicated to a placebo effect. The sample size is a major limitation of this study; only 5 VP subjects were examined; therefore external validity is extremely low (28). Jang et al. (22), in a randomized, double-blind sham-controlled study on 20 subjects with parkinsonism, showed that 10 Hz rTMS over M1 and dorsolateral prefrontal cortex could be effective for freezing of gait (a pivot symptom of VP). However, results from their study cannot be applied to VP subjects as the VP diagnosis criterion from this study was not explicitly stated, and therefore, the probability of misdiagnosis remains high. Our systematic review found another study (39) reporting treatment of VP with rTMS that showed promising results but ultimately it was not included in the analysis as the VP definition the authors used excluded subjects with good response to levodopa (40).

As for lumbar puncture therapy, a recent review by Korczyn (3) stated that "CSF drainage to treat patients with VP has produced positive results"; however, based on our systematic review only one study has explored the effect of lumbar puncture on VP (32). Previously reported studies focused on idiopathic normal pressure hydrocephalus (iNPH) (41–43), and while these two disorders share certain symptoms and radiological signs, VP has a different etiology and pathophysiology (42). Vizcarra et al. (6) pointed out that no clinical or radiological feature can accurately differentiate VP from iNPH. To evaluate if VP is responsive to lumbar puncture, pathological corroboration of vascular disease would require knowledge of the positive and negative predictive values of any method proposed to differentiate VP from iNPH in order to make clinical judgments as to potential treatments.

During the 2-year follow-up period of an open-label study, vitamin D (at a daily dose of 1,200 IU ergocalciferol) was proven to reduce the number of falls and hip fractures in VP patients as compared to those with PD. No potential mechanisms of action were explored, but vitamin D is theorized to play an active role in muscle strength. Limitations of the study include an absence of a placebo and age-matched controls. Due to methodological constraints, this study is not enough to confirm the effects of vitamin D on VP subjects. If more evidence is documented, vitamin D could be recommended as an adjuvant therapy to prevent complications of VP (24).

Medication used for secondary stroke prevention may be a suitable option for preventing the worsening of VP symptoms and improving its prognosis as they help to control vascular risk factors (3, 44). However, none of the studies included in our systematic review contained information on subjects taking medication for secondary stroke prevention or its influence on clinical response. To the best of our knowledge, there have been no clinical trials or cohort studies that focus on this issue in VP subjects. However, in the absence of definitive prospective controlled studies, it may be reasonable to extrapolate from studies and clinical results of treatment of stroke in general.

As previously stated, levodopa therapy is the most effective treatment for PD. According to our systematic review, it is also the most studied option for VP therapy. However, studies have determined that levodopa is a non-effective therapy (3) for VP subjects. This conclusion has been perpetuated by classic studies (45, 46) in which VP was poorly defined or not defined at all. Our meta-analysis revealed that approximately 30% of VP subjects do respond to levodopa therapy. We found high heterogeneity and initially guessed that this heterogeneity would present a problem due to the different criteria used for diagnosis in each study. However, surprisingly, heterogeneity was not explained by the diagnosis criteria but by the type of study. Regardless of the diagnostic criteria reported, the event-rate meta-analysis showed a low proportion of subjects responding to levodopa. Subjects diagnosed with Zijlmans' criteria showed the highest proportion of response to levodopa (OR 0.379, CI 95% 0.262–0.513; Supplementary Material); however, due to overlapping CI, no statistical difference was observed in comparison to other diagnostic criteria. Although our results show that the criteria used for diagnosis do not change the event-rate response, we highly recommend maintaining the actual tendency of diagnosing VP subjects with standardized criteria until more definitive knowledge is obtained. This would increase the external validity of future research and certainly will make results between studies more comparable.

In the revised studies, vascular lesions were found on external capsule, corona radiata, thalamus, pons, basal ganglia, and substantia nigra (Tables S1 and S2 in Supplementary Material). We decided to pool single photon emission computed tomography (SPECT) studies with the clinicopathological study in which nigrostriatal lesion was confirmed. Even though two different methods of assessing nigrostriatal integrity were used, sensitive analysis showed that excluding the clinicopathological study did not change the outcome. Our results showed that VP subjects with lesion in the nigrostriatal pathway are 15 times more likely to respond to levodopa than VP subjects without these lesions. Although the OR is large, we should still take into account that the proportion of the VP subjects with nigrostriatal lesion that respond to levodopa is just above 50%.

Previously, Vizcarra et al. reported that for a true VP diagnosis, ischemic or hemorrhagic lesions in the nigrostriatal pathway were needed (6). Otherwise, diagnoses made by clinical presentation and magnetic resonance imaging may have a certain degree of inaccuracy. Our results concur with Vizcarra et al., a dopamine transporter deficiency measured with SPECT, predicts a much better response to levodopa therapy, and therefore dopamine

transporter deficiency can be a good predictor of levodopa response in VP patients. However, the negative predictive value cannot be ascertained. Consequently, the process of clinical diagnosis and determination of treatment also has to consider the consequences of failure to use levodopa due to a false negative study.

Finally, we found that the probability of responsiveness to levodopa for a VP subject is 0.018 times the probability of a PD subject. This result could also be expressed in the following way: for every 55 PD subjects that respond to levodopa only one VP subject responds to it. This disparity in responsiveness can be useful in the differential diagnosis of VP and PD, but should be read with caution: even when VP subjects' probability of responding is very low compared to PD subjects, approximately 30% of the VP subjects do respond to levodopa. The last statement should discourage movement disorder specialists from using lack of levodopa response to pinpoint VP. Interestingly, this disparity may suggest different pathophysiological mechanisms underlying the clinical phenomena of VP, particularly as it related to the presence and absence of nigral involvement, compared to idiopathic PD. It is important to highlight that vascular risk factors are more prevalent in the aging population. As a great proportion of PD subjects have vascular risk factors and even radiological evidence of lacunar strokes (4), part of making a correct differential diagnosis between VP and PD should be an assessment of the causal role of the vascular lesions in the clinical syndrome.

Our study has several limitations: first, only observational studies were found, so potential bias and inaccurate conclusions are possible concerning the efficacy of treatments; also, most of the studies retrieved had a small sample size (14–47 subjects per study); therefore, inherent bias may be implicit. Second, we did not search for unpublished data and only papers published in English were considered; therefore, publication bias cannot be ruled out. Third, as not all studies reported mean equivalent doses of medication, a further meta-regression analysis of dose–response could not be done; consequently, we could not assess if dosage has any influence on clinical response. Fourth, although it has been reported that VP subjects on average have a greater age than PD subjects (16, 21, 22, 29, 35), most studies included in this analysis do not report the specific age of the responsive subjects. As with age, genre differences in levodopa responsiveness have been reported in studies (16, 20, 25). However, the specific genre of responsive subjects was not reported in the studies that met the criteria of this review; thus it could not be analyzed if a subject's age or genre has any interaction with clinical response rates of treatment. Finally, even with standardized criteria as those of Ziljmans' and Winikates', the diagnosis of VP is still inexact; its true prevalence and incidence are unknown; therefore, any recommendations given in this review must take into account the limitations of VP diagnosis. To the best of our knowledge, no study has aimed to measure the positive and negative predictive values of standardized criteria.

We conclude that further investigation of diagnostic procedures is needed to provide positive and negative predictive values for this neurological disorder. Additionally, randomized placebo-controlled clinical trials of available therapeutic interventions using a clear definition of VP are urgently needed to be able to provide optimal care for VP subjects and avoid the consequences of false positive and false negative diagnoses. Although to date there is insufficient evidence in the literature to make any recommendations as to the treatments for VP, the small number of subjects that are responsive to levodopa certainly merits a trial use of this drug to ascertain individual responsiveness. Also, adjuvant therapy with vitamin D and rTMS may be promising. Despite the limitations of differing diagnostic criteria, the results of this meta-analysis would seem to indicate that responsiveness to levodopa is not a reliable determinant for a differential VP/PD diagnosis.

AUTHOR CONTRIBUTIONS

AM-P, GV, JS-P, and OA-C were involved in conceptualization, design, and interpretation of results. AM-P and GV were in charge of coding, data extraction, and statistical analysis. OA-C: data output analysis. All four authors wrote and approved this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fneur.2017.00481/full#supplementary-material.

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Implications of DNA Methylation in Parkinson's Disease

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It has been 200 years since Parkinson's disease (PD) was first described, yet many aspects of its etiopathogenesis remain unclear. PD is a progressive and complex neurodegenerative disorder caused by genetic and environmental factors including aging, nutrition, pesticides and exposure to heavy metals. DNA methylation may be altered in response to some of these factors; therefore, it is proposed that epigenetic mechanisms, particularly DNA methylation, can have a fundamental role in gene–environment interactions that are related with PD. Epigenetic changes in PD-associated genes are now widely studied in different populations, to discover the mechanisms that contribute to disease development and identify novel biomarkers for early diagnosis and future pharmacological treatment. While initial studies sought to find associations between promoter DNA methylation and the regulation of associated genes in PD brain tissue, more recent studies have described concordant DNA methylation patterns between blood and brain tissue DNA. These data justify the use of peripheral blood samples instead of brain tissue for epigenetic studies. Here, we summarize the current data about DNA methylation changes in PD and discuss the potential of DNA methylation as a potential biomarker for PD. Additionally, we discuss environmental and nutritional factors that have been implicated in DNA methylation. Although the search for significant DNA methylation changes and gene expression analyses of PD-associated genes have yielded inconsistent and contradictory results, epigenetic modifications remain under investigation for their potential to reveal the link between environmental risk factors and the development of PD.

Keywords: 5-methylcytosine, DNA methylation, epigenetics, folate, alpha-synuclein, neurodegeneration, Parkinson disease

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INTRODUCTION

Parkinson's disease (PD) is the second most common chronic neurodegenerative disease in the elderly population. The motor symptoms that characterize PD are bradykinesia, tremor, rigidity, and postural instability, together with non-motor symptoms such as depression, anxiety, sleep disorders and cognitive dysfunction. These symptoms lead to severe impairment of the quality of life for the PD patient (Frucht, 2004). Pathological analyses of *post-mortem* brains have shown

Lewy bodies, which are abnormal protein aggregates found within nerve cells, and a progressive loss of *substantia nigra* dopamine neurons (Iacono et al., 2015). Among the molecular mechanisms suggested to cause PD are cellular oxidative stress and autophagy. Pesticide exposure, use of well water, heavy metal exposure, and industrialization are some of the environmental factors that contribute to the development of PD (Willis et al., 2010a,b).

Over recent years, extensive genetic screening of PD families has aimed at identifying mutations associated with the disease that would give a deeper insight into the molecular mechanisms underlying the PD pathology. Genetic studies identified several genomic risk *loci* associated with familial PD, such as *PARK1-15* and other related genes (Masliah et al., 2000; Cheon et al., 2012). Additionally, other genes, including *LRRK2*, *SNCA*, *MAPT,* and *GBA*, have been associated with sporadic PD (Coppede, 2012). Even though there is evidence that PD can be caused sporadically by familial genetic mutations (causal mutations), such as in *alpha-synuclein* (*SNCA*) or *Parkin*, it is more likely that in most patients the disease develops as a consequence of the combination of mutations in multiple PD-associated genes and environmental risk factors.

In addition to the genetic component involved in the development of many disorders (acquired mutations in one gene or a group of genes), epigenetic mechanisms have been found to contribute significantly to their development. Epigenetic factors are chemical modifications of chromatin or its regulatory proteins that do not change the underlying genomic sequence. These modifications can modulate gene expression, allowing differentiation into different cellular phenotypes by driving tissue-specific expression patterns. These changes include DNA methylation, post-translational modifications of histones, chromatin remodeling, as well as small and long non-coding RNAs (Turner, 2007).

Epigenetic regulation of biological processes is known to be essential during embryonic development, early brain programming, neurogenesis and brain plasticity (Yao et al., 2016). Therefore, it is not surprising that epigenetic deregulation can be critical for the onset of various neurodegenerative diseases, such as PD (Ammal Kaidery et al., 2013). Previously, a comprehensive genomic study identified several PD risk *loci* in cerebellum and frontal cortex of PD brains, including *PARK16*, *GPNMB*, and *STX1B* genes, that were associated with differential DNA methylation at proximal CpG sites (International Parkinson's Disease Genomics Consortium (IPDGC) and Wellcome Trust Case Control Consortium 2 (WTCCC2), 2011).

Importantly, as there is currently no animal model that mimics PD; human brain is still the model used to study epigenetic changes. However, as PD brain tissue is predominantly analyzed *post-mortem*, this data cannot provide information about disease-progressive alterations, the extent of variations induced by previous therapeutic treatments and the occurrence of potential PD biomarkers. Access to brain samples for research is limited, so the focus has been on finding a more easily accessible tissue, such as peripheral blood, as a surrogate for brain tissue. For this purpose, a genome-wide study examined DNA methylation changes in PD patients by collecting fresh *post-mortem* brain and blood samples from PD patients and age-matched healthy subjects (Masliah et al., 2013). This comparison revealed that both tissues exhibited highly similar global DNA methylation patterns. Accordingly, Masliah et al. (2013) identified groups of genes with either increased or decreased DNA methylation in both PD brain and blood samples. Importantly, analysis of DNA methylation profiles of blood clearly distinguishes PD patients from healthy subjects or subjects with other disorders. These results suggest that, firstly, peripheral blood may be a valid surrogate for brain tissue samples, and secondly, epigenetic changes could potentially serve as biomarkers for the diagnosis of PD. Early biomarkers could improve the prognosis of PD by facilitating the initiation of rational treatment before significant neurological damage takes place. Here, we discuss the current evidence for DNA methylation changes in PD, including the involvement of nutrition and environmental factors.

THE ROLE OF DNA METHYLATION IN DISEASE AND AGING

DNA methylation is the most studied epigenetic modification, one that has been investigated in almost all pathologies. In mammals, DNA methylation takes place predominantly in the context of CpG dinucleotides (Ehrlich and Wang, 1981). While overall the genome is widely depleted of CpGs, CpG islands are regions of high CpG content (Takai and Jones, 2002; Wu et al., 2010). CpG islands are characteristic for more than 60% of all promoters of protein-coding genes. Whereas on a genome-wide level up to 70–80% of all CpG sites are methylated, CpG islands are mostly devoid of DNA methylation (Bird, 2002; Edwards et al., 2010). Adjacent to a CpG island, 2 kilo base pairs (kb) up- and down-stream, are its so-called CpG shores (Irizarry et al., 2009). The presence of DNA methylation, at least at gene promoters and regions of repetitive sequences, is linked to chromatin silencing (Hsieh, 1994; Siegfried et al., 1999). Two principal mechanisms, which are not mutually exclusive, are thought to explain the repressive effect of DNA methylation on gene repression (Bird and Wolffe, 1999; Klose and Bird, 2006). First: DNA methylation interferes with the recognition of transcription factor (TF) binding sites and thereby impairs gene activation (Domcke et al., 2015). Second: DNA methylation is recognized by specific Methyl-CpG binding proteins, such as MeCP2, that recruit co-repressor protein complexes and thereby mediate silencing (Nan et al., 1998). Aberrant methylation patterns at CpG islands and shores have been linked to human disease, including multiple cancers (Ohm et al., 2007; Irizarry et al., 2009; Berman et al., 2011; Manjegowda et al., 2017).

In developing embryos and germ cells, DNA methylation patterns are first established by *de novo* DNA methyltransferases (DNMTs), DNMT3A and DNMT3B. After this, DNA methylation is maintained during DNA replication by DNMT1, which localizes to the replication fork during S-phase where it binds to hemimethylated CpGs (Jones and Liang, 2009; Jung et al., 2017). In 2009, ten-eleven translocation (TET) enzymes were identified that can reverse DNA methylation, by oxidization of 5-methyl cytosine (5mC) to 5-hydroxymethyl

cytosine (5hmC) (Tahiliani et al., 2009; Pastor et al., 2013). 5hmC can be lost passively by dilution during replication or be actively removed by subsequent oxidative reactions catalyzed by TET proteins that result in the formation of 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) as intermediates (Ito et al., 2011). Finally, the thymine DNA glycosylase (TDG)-mediated base excision repair (BER) replaces the methylated site by an unmodified cytosine (He et al., 2011; Kohli and Zhang, 2013).

Importantly, it appears that the 5hmC modification is not only an intermediate of 5mC demethylation, but has been considered to be an epigenetic mark in itself. Although the exact role of 5hmC is still being studied intensively, it seems to have a distinct function from that of 5mC. Particularly in neuronal cells, DNA hydroxymethylation was found to be enriched in gene bodies of actively transcribed genes (Mellen et al., 2012; Hahn et al., 2013). Given the relative abundance of DNA hydroxymethylation in the brain and its apparent role in normal brain maturation and memory formation (Szulwach et al., 2011; Kaas et al., 2013; Lister et al., 2013; Kinde et al., 2015), it has been implicated in the onset and progression of several neurodegenerative disorders (Villar-Menendez et al., 2013; Wang et al., 2013; Condliffe et al., 2014; Coppieters et al., 2014). Although not as widespread as 5mC and 5hmC, there is emerging evidence that cytosine methylation also exists outside of the sequence context of CpG sites (non-CpG methylation: CpA, CpT, and CpC) and appears to be most common in embryonic stem cells (Lister et al., 2009) and adult brain tissue (Varley et al., 2013; Guo et al., 2014). Non-CpG methylation occurs postnatally during the primary phase of neuronal maturation and may play a role in transcriptional repression (Lister et al., 2013; Guo et al., 2014). However, as it is technically challenging to target this modification *in vivo* without altering CpG methylation in the process, extensive research is still required to elucidate the distinct biological function of non-CpG methylation.

Genetically, aging is characterized by distinct alterations that take place at the chromatin level. These include telomere shortening, increased genome instability and changes of epigenetic signatures, such as DNA methylation patterns (Lopez-Otin et al., 2013). Age-related remodeling of DNA methylation comprises events of both hypo- and hypermethylation (Maegawa et al., 2010; Jung and Pfeifer, 2015). DNA hypomethylation happens globally at CpG sites outside of CpG islands (Christensen et al., 2009; Heyn et al., 2012; Day et al., 2013), while DNA hypermethylation affects mostly CpG islands in promoters of genes, which are frequently involved in development and differentiation (Christensen et al., 2009; Rakyan et al., 2010). Therefore, over time the accumulation of epimutations, which are heritable changes of gene activity mediated by epigenetic alterations, are believed to contribute to genomic instability just as genetic mutations do.

Interestingly, the methylation states at specific CpG *loci* can be consulted as epigenetic biomarkers to reliably predict the human chronological age (Horvath, 2013; Weidner et al., 2014). Moreover, twin studies determined that in each human, the age-dependent aggregation of distinct epigenetic changes, termed 'epigenetic drift,' is thought to be influenced predominantly by environmental factors (Fraga et al., 2005; Tan Q. et al., 2016). The individual differences in exposure to these factors are suspected to contribute to variation in disease susceptibility, onset, progression, etiopathology, treatment response and disease outcome. Taken together, changes in DNA methylation patterns and their effects on chromatin and gene expression appear to add increasingly to our understanding of age-related diseases, including PD. In the following, we will summarize and discuss the current evidence of DNA methylation changes at candidate genes that could be related to the development of PD.

DNA METHYLATION AND PD: ANALYSIS OF THE *SNCA* GENE

The *SNCA*p.Ala53Thr mutation, described in 1997, was the first genetic cause of PD identified (Polymeropoulos et al., 1997). This missense mutation provided the first link between *SNCA* and familial PD after its identification in a family from Southern Italy. The respective gene product, the SNCA protein, was discovered almost simultaneously (Spillantini et al., 1997). At the molecular level, SNCA aggregation contributes majorly to the formation of Lewy bodies, a hallmark of PD pathology. In addition to genetic mutations, also *SNCA locus* amplifications (duplications, triplications) have been found as a cause of familial PD (Singleton et al., 2003; Chartier-Harlin et al., 2004). *SNC*A point mutations, as well as gene multiplications and overexpression, are all thought to play a causal role in the formation of Lewy bodies (Narhi et al., 1999; Masliah et al., 2000). That *SNCA* gene dosage is critical for the development of PD, was further supported by mouse models with neuronal expression of wild-type *SNCA* (Masliah et al., 2000; Janezic et al., 2013). These transgenic mice revealed PD-like loss of dopaminergic neurons, protein aggregate formation, and motor impairments.

As gene dosage can be changed not only by gene amplification, but also by gene regulation, DNA methylation was considered as a potential mechanism that could be involved in the deregulation of *SNCA* in the case of PD. Accordingly, sequence analysis of the promoter region of the *SNCA* gene led to the identification of two CpG islands (Matsumoto et al., 2010). The first, CpG-1 is located in the first exon but does not overlap with the coding region of *SNCA*, and the second, CpG-2 is located in the first intron. In luciferase reporter assays the promoter activity of sequences containing CpG-2 was indeed strongly reduced by *in vitro* DNA methylation prior to cell transfection (Jowaed et al., 2010; Matsumoto et al., 2010). Furthermore, treatment of SK-N-SH cells with a DNA methylation inhibitor resulted in a reduction of CpG-2 methylation and a significant increase of *SNCA* mRNA and protein levels (Jowaed et al., 2010). These data supported the idea that DNA methylation at least at the intronic CpG-2 island could control *SNCA* gene activity. In fact, several studies analyzing the DNA methylation levels in samples of PD patients compared to controls confirmed a hypomethylation of intron 1 that coincides with the second *SNCA* CpG island. Using brain samples a significant demethylation of intron 1 was reported in the *substantia nigra pars compacta* (SnPC) of PD patients which could explain increased *SNCA* expression (Jowaed et al., 2010; Matsumoto et al., 2010). Taking into account the

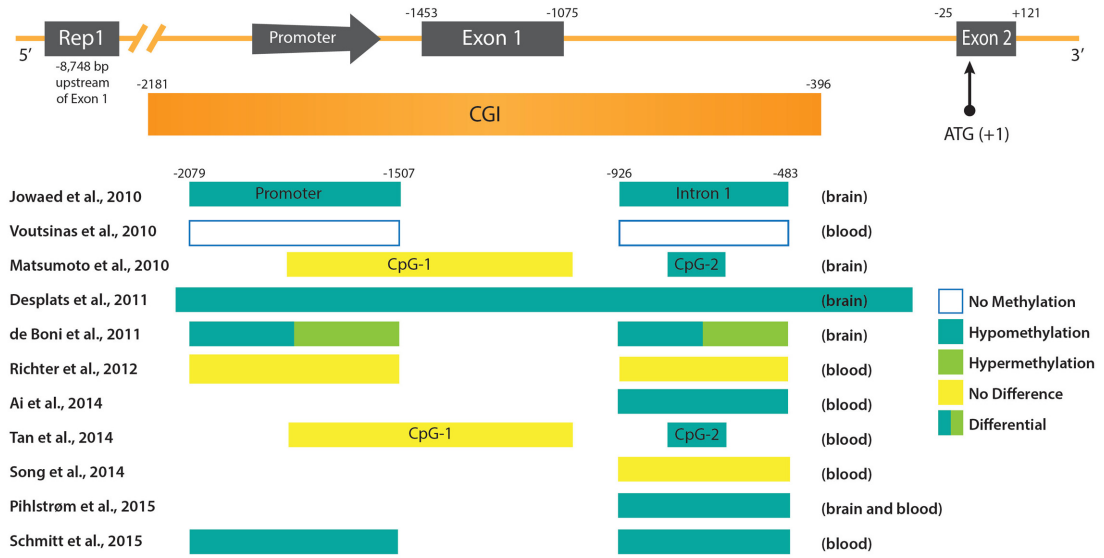

FIGURE 1 | DNA methylation patterns in PD at the promoter region of the *SNCA* gene. **(Upper)** The promoter region and 5′ end of the *SNCA* gene containing the core promoter and the two first exons of *SNCA* (Exon 1 and Exon 2) are depicted. Exon 2 contains the ATG start codon of the open reading frame (ORF). Upstream of the promoter the location of the polymorphic microsatellite sequence Rep1 is shown. Below the scheme of the *SNCA* gene the CpG-rich region is depicted as an CpG island (CGI). Positions of regulatory sequences (promoter and CGIs), as well as exons and introns are given at the top in reference to the ATG start codon. **(Lower)** Regulatory sequences and intronic regions analyzed in various studies (see text for more detail) are depicted as boxes. The references are given on the left, and the DNA methylation status and source of PD samples on the right.

observation that DNA methylation patterns between blood and brain tissue show a strong correlation (Masliah et al., 2013), more recent studies analyzed peripheral blood samples of PD subjects instead of or in addition to *post-mortem* brain tissue. In agreement with the assumption that DNA methylation profiles in the brain could be potentially mirrored in blood cells, a recent study found *SNCA* promoter hypomethylation in both *post-mortem* cortex and peripheral blood samples (Pihlstrom et al., 2015). Another study reported hypomethylation of *SNCA* intron 1 in peripheral blood mononuclear cells of 100 sporadic PD subjects (Ai et al., 2014). In 2015, the largest study carried out to date analyzing 490 peripheral blood samples of patients with sporadic PD, also revealed hypomethylation of *SNCA* intron 1. In contrast, *SNCA* methylation was found to be increased in PD patients who received higher L-dopa dosage. Accordingly, L-dopa led to a specific increment of DNA methylation of *SNCA* intron 1 in mononuclear cell cultures. Interestingly, the detection of DNMT1 in *post-mortem* brain tissue of PD patients and in *SNCA* transgenic mice uncovered that the amount of enzyme was strongly reduced in the nuclear fraction of neuronal cells (Desplats et al., 2011). Thus, sequestration of DNMT1 in the cytosol could explain the global, as well as the *SNCA* gene-specific, PD-dependent DNA hypomethylation, mechanistically.

In recent years, meta-analyses of genome-wide association studies (GWAS) on single nucleotide polymorphism (SNP) data of large PD case-control cohorts were conducted (Nalls et al., 2011, 2014; Sharma et al., 2012). These studies identified risk *loci* in both genes, previously not linked to PD pathology, and known key players, such as *SNCA*. Thereby, obtained results substantiated that there is a major genetic component contributing to the susceptibility to PD. But additionally, inter-individual genetic variants can frequently be associated with DNA methylation differences at distinct CpG sites and are defined in statistical analyses as methylation quantitative trait *loci* (mQTLs). Two recent studies investigated the relationship between genetic variation and CpG methylation in the human brain (Gibbs et al., 2010; Zhang et al., 2010). In case of the *SNCA* gene, three independent studies noted that the genotype SNP rs3756063 showed a significant correlation with the DNA methylation state of *SNCA* intron 1 both in brain and blood PD samples (Pihlstrom et al., 2015; Schmitt et al., 2015; Wei et al., 2016). However, it should be noted that an association between rs3756063 and the *SNCA* mRNA expression could not be found (Pihlstrom et al., 2015; Wei et al., 2016). Furthermore, another association could be established between the *SNCA* DNA methylation levels and the Rep1 polymorphism (Ai et al., 2014). In contrast to rs3756063, Rep1 is a complex microsatellite repeat polymorphism located approximately 10 kb upstream of the *SNCA* transcription start site (**Figure 1**). Its longest 263 bp allele has previously been associated with sporadic PD (Maraganore et al., 2006). In agreement with an elevated PD risk, genotypes carrying the 263 bp allele showed the strongest *SNCA* intron 1 hypomethylation (Ai et al., 2014). Experiments in transgenic mice suggested a *cis*-regulatory effect of the Rep1-length regulating *SNCA* transcription, whereby homozygosity of the expanded 263 bp allele correlated with the highest gene expression (Cronin et al., 2009). Controversially, recent data obtained by the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technique, to edit the genome Rep1 *locus* in human embryonic stem cell-derived neurons, contradict

the enhancer function of the repeat sequence and do not detect a correlation between *SNCA* expression and Rep1-length (Soldner et al., 2016). Likewise, the interdependency between DNA methylation alterations in the *SNCA locus* and genetic variants, is not understood mechanistically, neither in case of Rep1 nor rs3756063. Genetic variation does not only impact DNA methylation, but also dictates differences in binding of TFs in individuals on a genome-wide level (Kasowski et al., 2010). Therefore, it will be a great challenge to unravel the molecular mechanisms behind these associations, as they are expected to be connected with each other in a complicated network.

Although the studies cited above suggest DNA hypomethylation of the *SNCA* promoter region in PD patients, discrepancies with other findings exist. For example, one study revealed variations of DNA methylation levels at the *SNCA* gene in different brain regions. Both hypomethylation, as well as hypermethylation, were detected in various Lewy body disease/PD stages in both the promoter region and intron 1 (de Boni et al., 2011). Another recent study found no intron 1 hypomethylation of *SNCA* in a limited number of PD patients (Guhathakurta et al., 2017). Moreover, the analysis of blood samples from 43 PD subjects provided no evidence for DNA methylation changes within the *SNCA* promoter region (Richter et al., 2012), just as another study comprising blood leukocyte samples of 50 PD patients (Song et al., 2014). However, it should be noted, that in the latter two studies (Richter et al., 2012; Song et al., 2014) 10 times less subjects participated compared to the analysis of Schmitt et al. (2015) that detected differential DNA methylation at the *SNCA* gene.

In light of the inconsistencies concerning the relevance of DNA hypomethylation at the *SNCA* intron 1 in association with PD, additional studies will be needed to resolve these doubts. Also, even if *SNCA* intron 1 hypomethylation can be consistently confirmed in PD, similar DNA patterns were also found in both Dementia with Lewy Bodies (DLB) (Funahashi et al., 2017) and AD (Yoshino et al., 2016). Therefore, it is conceivable that this DNA methylation change would not serve as a specific biomarker for PD, but a more general one for all Lewy body pathologies.

DNA METHYLATION PATTERNS AT PD-ASSOCIATED GENES

In the following section, we will give an overview of what is known so far about the epigenetic signatures of DNA methylation at genes that were found to be associated with the development of PD (compare **Table 1**).

Due to its relation to *SNCA* and its abundance in neurofibrillary lesions of patients with AD, the *beta-synuclein* (*SNCB*) gene has also been considered as a possible player in PD. Interestingly, SNCB inhibits the generation of SNCA fibril aggregation *in vitro* (Park et al., 2003) and therefore, may play a neuroprotective role (Vigneswara et al., 2013). Furthermore, *SNCB* and *SNCA* have similar expression levels in nervous system tissue samples (Maroteaux et al., 1988). Moreover, SNCA and SNCB associate *in vitro* whereby SNCB may protect SNCA from aging-related protein damage (Vigneswara et al., 2013). However, to date, the specific role SNCB plays in PD has not been elucidated. A DNA methylation study carried out in PD samples to examine the *SNCB* gene found its promoter to be unmethylated in *post-mortem* brain from PD and PD-Dementia samples. Additionally, bisulfite sequencing of the *SNCB* promoter in four pure diffuse Lewy body pathology cases did not reveal methylated cytosines along the CpG island (Beyer et al., 2010).

Post-translational citrullination (deimination) is mediated by peptidyl arginine aminases (PADs) and has been implicated as an unusual pathological trait in neurodegeneration and inflammatory responses in multiple sclerosis, AD and prion diseases (Jang et al., 2013). Alterations in the expression of these proteins have also been seen in *post-mortem* samples taken from different brain areas of PD subjects (Nicholas, 2011). In thymus samples from multiple sclerosis patients, the promoter of *PAD2* (peptidyl arginine deaminase type II) was reported to be hypomethylated (Sokratous et al., 2016). In contrast, white matter from PD, AD, or Huntington disease patients showed that *PAD2* was not hypomethylated (Mastronardi et al., 2007). DNA methylation analysis of the tumor necrosis factor alpha (TNF-alpha) gene, another PD-associated gene, showed a significantly lower methylation level comparing DNA from the SnPC to DNA from brain cortex. However, this difference could not be linked to PD as it was observed in both PD subjects and controls (Pieper et al., 2008). In another study, the *UCHL1* promoter from *post-mortem* frontal cortex samples was analyzed, and no differences in the percentage of CpG methylation between PD cases and controls were found (Barrachina and Ferrer, 2009).

Behrens et al. analyzed the *ATP13A2* promoter region from four PD subjects with Kufor-Rakeb syndrome, a rare Type 9 juvenile PD that is linked to a mutation in the *ATP13A2* gene (Behrens et al., 2010). No significant correlation between DNA methylation changes of the hypomethylated promoter and Kufor-Rakeb syndrome juvenile PD progression was found. Another study considered the known association of *Parkin* (*PARK2*) gene mutations with autosomal recessive juvenile PD. Samples from 17 PD subjects with heterozygous *Parkin* mutations, as well as 17 PD subjects without *Parkin* mutations, were compared to samples from 10 normal subjects. No significant differences in DNA methylation at CpG sites among these three groups were found, suggesting that a DNA methylation-related mechanism involving the *Parkin* gene was unlikely to play a role in the pathogenesis and development of this type of PD (Cai et al., 2011). A recent study compared the DNA methylation status of the *PARK2* promoter region in 5 *post-mortem* brain samples taken from *substantia nigra*, cerebellum, and occipital cortex (De Mena et al., 2013). In agreement, with previous results (Cai et al., 2011) no differential DNA methylation of *PARK2* was seen (De Mena et al., 2013).

The expression of clock genes, which are components of the circadian clock, is altered in leukocytes from patients with PD (Cai et al., 2010). With this in mind, a study was recently carried out in which DNA methylation status of the clock genes *PER1*, *PER2*, *CRY1*, *CRY2*, *Clock*, *NPAS2*, and *BMAL1* was measured in genomic DNA isolated from blood samples

TABLE 1 | DNA methylation status of PD associated genes.

Gene	Alias	Location	DNA methylation	Reference
PAD2	Peptidyl arginine deiminase 2	1p36.13	No difference	Mastronardi et al., 2007
ATP13A2	*PARK9*	1p36.13	No difference	Behrens et al., 2010
DJ-1	*PARK7*	1p36.23	No methylation	Tan Y. et al., 2016
NPAS2	Neuronal PAS2	2q11.2	Hypomethylation	Lin et al., 2012
UCHL1	Ubiquitin C-terminal hydrolase L1	4p13	No difference	Barrachina and Ferrer, 2009
PGC1-α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	4p15.2	Hypermethylation	Su et al., 2015
TNF-α	Tumor necrosis factor-alpha	6p21.33	No difference	Pieper et al., 2008
PARK2	Parkinson juvenile disease protein 2, *Parkin*	6q26	No difference	Cai et al., 2011
			No difference	De Mena et al., 2013
CYP2E1	Cytochrome P450-J	10q26.3	Hypomethylation	Kaut et al., 2012
NOS2	Nitric oxide synthase 2	17q11.2	Hypomethylation	Searles Nielsen et al., 2015
MAPT	Microtubule associated protein Tau	17q21.31	Differential methylation	Coupland et al., 2014
FANCC/TNKS2	Fanconi anemia group C protein/tankyrase 2	9q22.32/10q23.32	Differential methylation	Moore et al., 2014
PARK16/GPNMB/STX1B	*PARK16*/glycoprotein Nmb/syntaxin 1B	1q32/7p15.3/16p11.2	Differential methylation	International Parkinson's Disease Genomics Consortium [IPDGC], and Wellcome Trust Case Control Consortium 2 [WTCCC2]
Genome-wide	Top 30 differentially methylated genes: *KCTD5, VAV2, MOG, TRI M10, HLA-DQA1, ARHGEF10, GFPT 2, HLA-DRB5, TMEM9, MRI 1, MAPT, HLA-DRB6, LASS3, GSTTP 2, GSTTP.*		Hypermethylation	Masliah et al., 2013
	DNAJA3, JAKMIP 3, FRK, LRR C27, DMBX1, LGALS7, FOXK1, APBA1, MAGI2, SLC25A24, GSTT 1, MYOM2, MIR886, TUBA3E, TMCO3		Hypomethylation	

From left to right: Listed are genes, gene aliases, genomic locations (according to the latest GRCh38/hg38 assembly of the human genome available at the UCSC genome browser), DNA methylation status in PD and references.

of 206 PD subjects. DNA methylation was detectable in *CRY1* and *NPAS2* promoters whereas the remaining gene promoters analyzed were devoid of DNA methylation. Interestingly, DNA methylation frequency of the *NPAS2* promoter was significantly decreased in PD patients, suggesting that its promoter DNA methylation may contribute to the expression of clock genes in PD (Lin et al., 2012). This finding could be relevant, as sleep disturbance is a commonly reported early symptom of PD (Breen et al., 2014).

Another gene of interest for the analysis of epigenetic changes is the microtubule-associated protein tau (*MAPT*) gene, as a genetic association with PD has been noted in GWAS (Simon-Sanchez et al., 2009). When 28 *post-mortem* brain and 358 blood leukocyte samples were analyzed, higher DNA methylation in MAPT was detected in H1 haplotype versus H2 (Coupland et al., 2014). Notably, in previous studies the presence of the H1 haplotype was associated significantly with PD (Kwok et al., 2004; Zabetian et al., 2007; Refenes et al., 2009). Additionally, DNA hypermethylation of the MAPT gene was observed in the cerebellum, but not in putamen from PD subjects where the MAPT gene was hypomethylated as compared with controls (Coupland et al., 2014).

DNA hypermethylation of the peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1α) promoter was reported in a sample of sporadic PD *substantia nigra* samples compared to 10 age-matched controls (Su et al., 2015). Recently, *PARK7* (*DJ-1*) DNA methylation was analyzed in peripheral blood leukocytes in PD subjects and controls. In contrast to the hypermethylated PGC-1α promoter (Su et al., 2015), they found the CpG-1 and CpG-2 islands of *PARK7* to be unmethylated in both PD and the negative control group (Tan Y. et al., 2016).

To detect further PD associated DNA methylation variations, an epigenome-wide association study was done to analyze DNA methylation patterns in putamen samples from *post-mortem* brain tissue of six PD patients. DNA methylation levels were quantitatively determined at 27,500 CpG sites representing 14,495 genes. This analysis revealed decreased DNA methylation at the cytochrome P450 2E1 (*CYP2E1*) gene, together with increased expression of the respective *CYP2E1* messenger RNA, suggesting that this cytochrome gene may contribute to PD susceptibility. In another epigenome-wide association study, conducted to reveal prioritized genes and pathways with statistically significant DNA methylation changes in PD, followed by a subsequent replication analysis

of top-ranked CpG sites, single CpG sites of *FANCC* and *TNKS2* showed significant differential methylation between PD cases and controls (Moore et al., 2014). In total, 20 unique genes were identified with a sizable difference in DNA methylation.

Despite the lack of conclusive evidence for the involvement of DNA methylation in the epigenetic regulation of many PD-associated genes, the search for other PD-associated genes and their DNA methylation status is ongoing. Undoubtedly, the significance and consistency of results of genomic DNA methylation in promoter regions of blood samples in comparison with brain samples need to be tested further. Unfortunately, findings from studies searching for PD-specific DNA methylation signatures at the *SNCA* gene and other PD-associated genes are still inconsistent concerning the clinical significance and specificity of DNA methylation changes in PD. Most importantly, experimental evidence that directly links DNA methylation changes in PD to the deregulation of these genes is still missing. Thus, the importance of differential DNA methylation for molecular mechanisms contributing to the development of PD remains to be investigated in future studies.

NUTRITIONAL FACTORS AND THEIR IMPLICATIONS FOR PD

DNA Methylation and Folate Deficiency

In recent years, several studies have attempted to pinpoint an interrelation between DNA methylation and folate. However, a significant association between DNA methylation levels and folate status could not always be consistently replicated (Waterland and Jirtle, 2003; Waterland et al., 2006; Steegers-Theunissen et al., 2009; Tobi et al., 2009; Shin et al., 2010). Apart from folate, many other nutrients are known to play key roles in one-carbon metabolism and DNA methylation. More accurate studies that analyze the contribution of other nutrients involved in DNA methylation and gene-diet interactions for PD risk are necessary.

The extent of DNA methylation in the cell is directly associated with the physiological level of SAM, the major methyl donor for DNA methylation, and SAH, the demethylation product of SAM and an inhibitor of DNA methyltransferases. The ratio SAM/SAH is interpreted as the methylation potential and is determined by the homocysteine concentration. The latter is considered a biomarker of folate deficiency, as it is dependent on 5-methyl tetrahydrofolate (THF) availability in the one-carbon metabolism. The physiologic levels of homocysteine and subsequent methylation potential are determined primarily by the dietary intakes of methionine, folate, B12 vitamin and other nutrients.

Importantly, elevated levels of homocysteine may have a toxic effect on dopaminergic neurons (de Lau et al., 2005; Gorgone et al., 2012). Consistently, higher homocysteine concentrations have been reported in PD patients compared to controls. Moreover, serum homocysteine levels predict the SAM/SAH ratio in plasma, and the concentration of SAH shows a significant correlation with markers of neurodegeneration (Amyloid Precursor Protein and SNCA). This evidence supports the use of total homocysteine and SAM/SAH ratio as biomarkers of the DNA methylation potential in patients with PD (Obeid et al., 2009). However, we still lack information about the direct effect of nutrient intake on genomic DNA methylation, especially regarding the combinatorial effects of nutrients with other factors, such as gene polymorphisms and/or therapeutic drugs.

One-Carbon Metabolism and Polymorphisms

The presence of SNPs in genes encoding enzymes and transporters involved in the folate metabolism, impair methyl group bioavailability and have been associated with altered blood concentrations of biochemical markers, including folate, vitamin B12 and homocysteine (Hazra et al., 2009; Tanaka et al., 2009; Liang et al., 2014). Importantly, some SNPs led to changes of homocysteine levels and were associated with differences in global DNA methylation levels (Wernimont et al., 2011). Other SNPs have also been associated with diseases, such as neural tube defects (Carter et al., 2011; Fisk Green et al., 2013; Ouyang et al., 2013; Liu et al., 2014) and different types of cancers (Curtin et al., 2007; Collin et al., 2009; Gibson et al., 2011; Levine et al., 2011; Metayer et al., 2011; Weiner et al., 2012).

The C677T variant (rs1801133), in the gene encoding the enzyme methylene-tetrahydrofolate reductase (MTHFR), is one of the most-studied SNPs occurring in components of the one-carbon metabolism. The base C677T substitution results in an amino acid change in the catalytic domain of the enzyme. This variation leads to a reduced protein stability and a 30% and 65% reduction of enzymatic activity in heterozygotes (CT) and homozygotes (TT), respectively (Rozen, 1997). Notably, the C677T variant has been previously reported to be associated with PD susceptibility (de Lau et al., 2005; Wu et al., 2013). A recent meta-analysis including data from fifteen studies (comprising 2690 PD cases and 8465 controls) did not find an appreciable difference in the general allelic frequency distribution of C677T between PD cases and controls (Zhu et al., 2015). However, in separate analyses that were stratified for ethnicity, a clear association was detected in Europeans (OR = 1.17), but not in Asians. Interestingly, this appears to be in line with the observation that the allelic frequency of the MTHFR C677T variant differs considerably between ethnic groups (Wilcken et al., 2003; Gueant-Rodriguez et al., 2006). Furthermore, this study confirmed that the T allele is an independent risk factor for increased homocysteine levels in PD patients (Zhu et al., 2015). In contrast, the results of a cohort study analyzing Chinese patients suggested that the A-T haplotype of A1298C, another common MTHFR variant, and C677T decreases the PD susceptibility (Yuan et al., 2016). The inconsistent findings for the association between C677T and PD may be explained by different genetic backgrounds, environmental factors or DNA methylation modulation.

OTHER NUTRITIONAL/ENVIRONMENTAL FACTORS AND DNA METHYLATION

Coffee Drinking

The risk for PD is ∼25% lower for coffee drinkers with a linear dose-response effect (Costa et al., 2010; Delamarre and Meissner, 2017). Caffeine is thought to act as an adenosine receptor antagonist, and to reduce inflammation and lipid-mediated oxidative stress (Farooqui and Farooqui, 2011; Kolahdouzan and Hamadeh, 2017).

Little is known whether DNA methylation changes can arise in response to distinct coffee consumption patterns. A recent study using blood tissue data of patients without PD found the methylation status of CpG sites located near genes previously linked to some familial forms of PD (*GBA*, *PARK2/Parkin*, and *PINK1*) associated with coffee consumption (Chuang et al., 2017). However, whether distinct DNA methylation levels at these CpG sites in coffee-drinkers are indeed protective against PD, remains to be further investigated.

Manganese

Manganese (Mn) is an essential element, but some industrial activities can result in exposure to high occupational and environmental Mn levels (Bowler et al., 2011, 2016). In the environment, Mn in drinking-water and foods may also contribute to toxic effects (ATSDR, 2012).

Exposure to excessive amounts of Mn may lead to adverse health outcomes, and evidence suggests that DNA methylation changes induced by Mn may play a relevant role. Regarding PD risk, gene activity of *PARK2* and *PINK1* was altered via DNA hypermethylation in dopaminergic human neuroblastoma SH-SY5Y cells upon Mn exposure (Tarale et al., 2016). Furthermore, mice exposed to $MnCl_2$ showed DNA hypo- and hypermethylation of different *loci* in *substantia nigra* (Yang et al., 2016). In human, the effects of Mn on parkinsonism via DNA methylation changes was assessed in welders' blood samples. Interestingly, subjects recently exposed to welding fume had lower *NOS2* gene DNA methylation than subjects retired from welding worksites. Also, an inverse association between duration of welding fume exposure and DNA methylation of a *NOS2* CpG site was observed (Searles Nielsen et al., 2015).

Endocrine Disruptors and Pesticides

It is proposed that other factors, such as endocrine disruptors or pesticide exposure, may play a role in modulating DNA methylation, although the evidence from studies with PD patients or animal models is still limited. Results from experimental, clinical, and epidemiological studies implicate exposure to endocrine disruptors with processes related to neurodegenerative diseases (Kajta and Wojtowicz, 2013; Preciados et al., 2016). Among these compounds, Bisphenol-A has been linked to lower levels of DNA methylation in cerebral cortex and hippocampus in mice (Kumar and Thakur, 2017). Several studies have shown an association with frequent pesticide exposure in men and late-onset PD (Delamarre and Meissner, 2017). Recent findings show that organochlorines exposure of hippocampal-primary cultures causes global hypomethylation of DNA (Wnuk et al., 2016).

Although research suggests that these and other environmental exposures can modify epigenetic signatures; important questions remain open. Therefore, studies in this field will provide new insights into PD pathologic processes, and consequently provide novel preventive and therapeutic intervention strategies.

CONCLUDING REMARKS AND PERSPECTIVES

Currently, there is a plethora of methods used for measuring DNA methylation (Kurdyukov and Bullock, 2016). To date the "gold standard" for the quantification of DNA methylation is still considered to be bisulfite sequencing. Nowadays this method is often used for genome-wide studies in combination with next-generation sequencing. However, the generation of bisulfite-converted DNA, and its subsequent use has often been described as technically challenging. Additionally, bisulfite conversion can lead to DNA fragmentation and can make amplification of long DNA regions difficult while resulting in chimeric products (Kurdyukov and Bullock, 2016). An easier method is needed that does not require bisulfite conversion, for example, an endonuclease digestion-based assay (historically the first technique utilized for studying DNA methylation), which can be applied at gene-specific *loci*, but is also compatible with whole genome methylation profiling. Determining a standardized method for quantifying DNA methylation at the same genomic regions of reported PD-associated genes would be ideal for clinical research. Another issue is finding a method that can efficiently detect 5hmC and distinguish it from 5mC, not only on a genome-wide level, but also at bp resolution. Techniques applied to analyze DNA methylation changes associated with PD have mostly not discriminated between 5mC and 5hmC. Particularly in the brain, it will be of great interest to unravel whether the latter DNA methylation mark exhibits disease-specific patterns that could serve as biomarkers. A recent study found an approximate two-fold increase of global DNA hydroxymethylation in the cerebellum of PD patients (Stöger et al., 2017). As this analysis lacks information about where these changes take place in the genome, further experiments will need to shed light on the cause, and examine whether elevated 5hmC levels contribute to PD onset or progression or PD is the reason for the aberrant hydroxymethylation. Approaches that can specifically detect 5hmC have been described, e.g., antibody-based techniques or oxidative bisulfite sequencing (Booth et al., 2012; Skvortsova et al., 2017). These efforts make it likely that methods that discriminate between 5mC and 5hmC will be on hand in the future.

For now, more epigenetic studies are required, particularly ones conducted in different populations, to expand the currently available database of DNA methylation in PD-associated genes. Importantly, a more accurate consensus needs to be reached on

the benefit of peripheral blood samples versus brain samples. For the DNA methylation status of a specific gene promoter, such as *SNCA* or other PD-associated genes to be authenticated as a reliable biomarker of PD status, a significant number of studies reporting consistent results will be needed. In this context, careful analysis of Levodopa treatment effects on *SNCA* DNA methylation offers the prospect that in the not-so-distant future a reliable DNA methylation biomarker in PD with high sensitivity and specificity will be available (Schmitt et al., 2015). With the growing interest in research on the interdependency between nutrition and epigenetics, in the future we will get a better understanding of what effects nutritional factors have on DNA methylation and what their involvement is in diseases like PD.

Epigenetic modifications may prove to be the missing link between environmental risk factors and the development of PD. Epigenetic variances between individuals could help us to explain the striking clinical differences observed in the age of onset and progression of sporadic PD, and may open the way for rational therapeutic intervention targeting DNA methylation modifications associated with this disorder.

AUTHOR CONTRIBUTIONS

EM-M, KM, AS-C, JS-P, PV-C, and OA-C wrote the manuscript.

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Research article

H1/H2 *MAPT* haplotype and Parkinson's disease in Mexican mestizo population

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Tau

ABSTRACT

Parkinson's disease (PD) is characterized by bradykinesia, resting tremor, rigidity and postural instability as well as early symptoms. Previous studies that evaluated the association between H1/H2 *MAPT* haplotype and PD were mostly conducted in European populations in which the H1 haplotype was a reported risk factor for PD. Despite those findings, some studies have suggested that the association may be ethnically dependent. Since studies conducted in Latin American population have been scarce, we genotyped the H1/H2 *MAPT* haplotype in Mexican mestizo population as part of a PD case-control study. DNA was extracted from peripheral blood leucocytes in 108 cases and 108 controls and detection of the H1/H2 haplotypes was achieved by determining the MAPT_238 bp deletion/insertion variant at intron 9 through end-point PCR followed by visual 3% agarose gel electrophoresis interpretation. We observed no-association between genotypes and PD risk [OR/CI (Odds ratio/95% Confidence Interval) of 1.60 (0.78–3.29) for H1/H2 genotype and 2.26 (0.20–25.78) for H2/H2]. No-association was maintained when stratifying our groups by central ($p = 0.27$) and northern regions ($p = 0.70$). Our data suggest that H1/H2 *MAPT* haplotype is not a risk factor to PD in our population.

1. Introduction

1.1. Pathological mechanisms

Parkinson's disease (PD) is the second most common neurodegenerative disease and is characterized by bradykinesia, resting tremor, rigidity and postural instability as well as early symptoms such as hyposmia, constipation and sleep disorders, among others [1]. Pathological mechanisms include the death of melanin and dopamine-producing neurons in the Substantia Nigra Pars Compacta (SnPC) and the loss of Neuromelanin (NM) as a post-mortem feature [2]. Reports also identified alpha-synuclein [3] and tau [4] aggregates in Lewy Bodies. While the presence of metal ions and mitochondrial reactive oxygen species (ROS) formation have also been demonstrated [5]. Genetic causes were found in familial and sporadic PD [6]. Microtubule Associated Protein Tau (*MAPT*) gene has been highly associated [7] and studies reported an over-representation of the MAPT H1 haplotype in various neurodegenerative disorders including progressive supranuclear palsy [8], Alzheimer's Disease (AD) [9], and PD [10].

1.2. MAPT H1/H2 structure

The *MAPT* locus contains two reported haplotypes: the directly oriented H1 and the inverted H2 [11–13]. The H1 and H2 haplotypes may be distinguished by identifying one or more of the eight reported single nucleotide polymorphisms (SNPs) in absolute linkage disequilibrium. Another characteristic of H2 is the presence of a 238 bp deletion within intron 9 (Fig. 1) [8].

1.3. MAPT H1/H2 associations

A copious amount of studies have been conducted to determine the possible association between H1/H2 haplotypes and PD with contradictory results. A recent meta-analysis determined that the *MAPT*_238 bp deletion/insertion might modulate the risk of PD [14]. Also, studies in Greek and Serbian populations reported association

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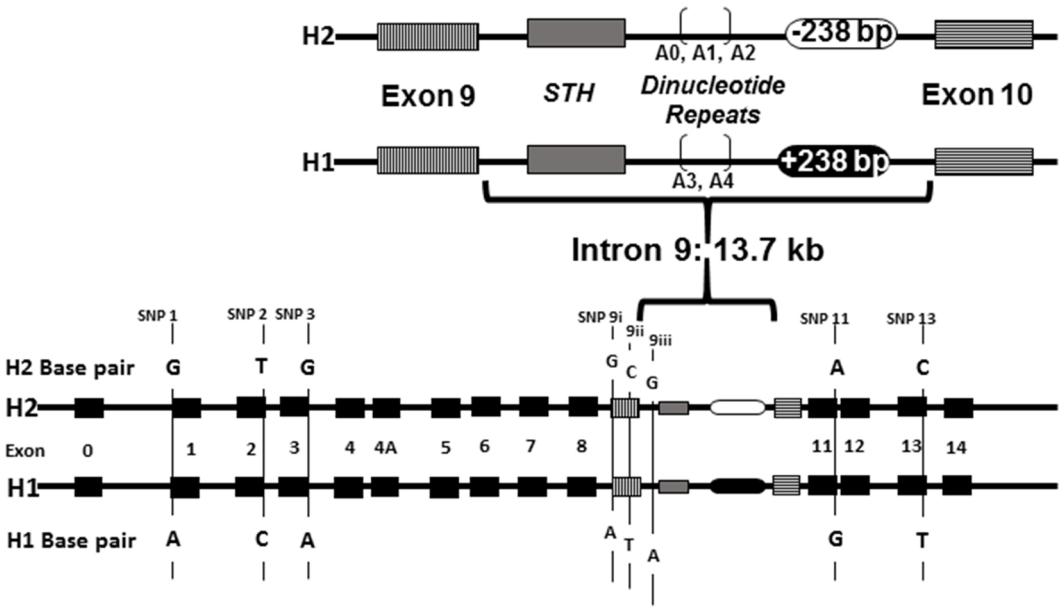

Fig. 1. Schematic representation of The *MAPT* region of interest. H1 and H2 contain 8 distinct SNPs (vertical lines with respective base pairs) and the MAPT_238 bp deletion (white oval)/insertion (black oval) variant at Intron 9. Black boxes indicate exons. Gray box indicates Saitohin (*STH*).

between H1 Haplotype and PD [15–17] and a report in UK Caucasian population found the H2 haplotype to be a protective factor [18]. However, reports in German, Indian, Greek and Finnish populations suggested that there is no correlation between H1/H2 haplotypes and PD [17,19,20]. The main goal of this work was to determine if there is an association between H1/H2 haplotypes and PD in a Mexican mestizo population.

2. Materials and methods

2.1. Information about participants

The subjects were recruited from three public hospitals. General Hospital Dr. Manuel Gea González in Mexico City (central region of Mexico), General Hospital 450 in Durango (northern region of Mexico) and General Hospital Santiago Ramón y Cajal in Durango (northern region of Mexico). PD was diagnosed using the UK Parkinson's Disease Society Brain Bank Diagnostic Criteria (UKPDSBB). Only those with late-onset disease (after 50 years of age) were included. The ethics committee from Dr. Manuel Gea González General Hospital authorized the study. Procedures were in accordance with the ethical standards of the Helsinki Declaration. A life-style interview was applied and written consent forms were signed prior to any intervention. Subjects were programmed for a fasting peripheral blood draw using the BD Vacutainer® collection system.

2.2. Genotyping methods

Samples were stored at 1°–6 °C. The DNA was extracted from peripheral blood leucocytes using the QIAamp DNA Blood Mini Kit® by QIAGEN® and quantified using a Thermo Scientific™ NanoDrop 2000 spectrophotometer. The H1/H2 haplotypes were determined analyzing the MAPT_238 bp deletion/insertion by PCR using the primer sequences GGAAGACGTTCTCACTGATCTG (forward) and AGGAGTCTGGCTTCA GTCTCTC (reverse). The 238 bp deletion was determined by the amplification of one distinct band at a size of 246 bp (H2/H2 haplotype); the amplification of two distinct bands (484 bp and 246 bp) corroborated the H1/H2 haplotype; and finally, the amplification of a 484 bp band only, corroborated the H1/H1 haplotype (Fig. 2).

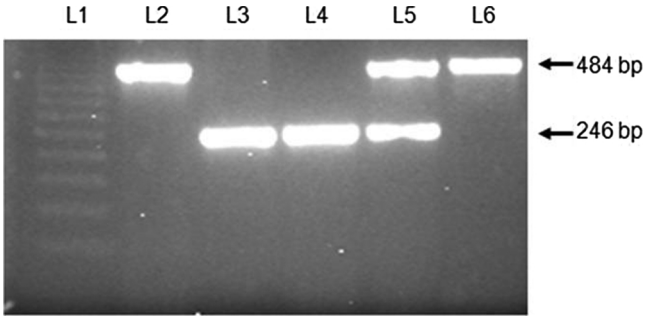

Fig. 2. Genotypification of H1/H2 haplotype using end point PCR (Genomic DNA in 3% agarose gel stained with ethidium bromide). L1, 50 bp DNA marker; L2 and L6, H1/H1 genotype; L3 and L4, H2/H2 genotype; L5, H1/H2 genotype.

2.3. Genotyping data analysis

For our genotypic analysis we utilized an on-line program provided by Institut Català d'Oncologia (https://snpstats.net/) as well as IBM SPSS Statistics for Windows (Version 21.0. Armonk, NY: IBM Corp.) for additional statistical analyses. For the purposes of this study p values < 0.05 were considered significant.

3. Results

3.1. General data about participants

In this work we included 108 cases and 108 controls. 39 paired cases resided in the central region versus 69 paired cases in the northern region. A total of 78 participants lived in the central region including Mexico City and surrounding states while 138 participants lived in the northern region including the city of Durango and rural areas of the state. The total age range of the participants was from 52 to 94 years with a mean age of 70.10 (± 9.16). Of these, 106 (49.1%) were women while 110 (50.9%) were men. For cases, the mean age at onset was 64.80 years (± 9.52). 18.51% of PD cases reported a family history of one or more first or second-degree relative(s) with PD. The mean evolution in years was 5.54 (± 4.11). Mean UPDRS III score was 43.00 (± 19.67), while total UPDRS score was 72.27 ± 33.29.

Table 1
Allelic and genotypic frequencies of H1/H2 haplotype and risk estimation to PD.

Haplotype	Controls n = 108	Cases n = 108	*p* value	OR (95% CI)	*p* value
H1	199 (0.92)	190 (0.88)	0.148*	1 (reference)	0.15
H2	17(0.08)	26 (0.12)		1.60 (0.84–3.04)	
H1/H1	92 (0.85)	84 (0.78)	0.363*	1 (reference)	0.36
H1/H2	15 (0.14)	22 (0.20)		1.60 (0.78–3.29)	
H2/H2	1 (0.01)	2 (0.02)		2.26 (0.20–25.78)	

* Pearson's Chi-squared is significant at $p \leq 0.05$.

3.2. Allelic and genotypic frequencies

The allelic and genotypic frequencies are shown (Table 1). Only one control and two cases presented the H2/H2 haplotype. No statistically significant differences were observed between groups in both, allelic (p = 0.148) and genotypic (p = 0.363) frequencies. The *odds ratio* estimation showed that neither the H2 allele (OR = 1.60, CI_{95} = 0.84–3.04) nor the H1/H2 (OR = 1.60, CI_{95} = 0.78–3.29) or H2/H2 (OR = 2.26, CI_{95} = 0.20–25.78) genotype are a risk factor for PD (Table 1).

3.3. Stratified allelic and genotypic frequencies

Subsequently, we stratified based on central or northern region from Mexico (Table 2). When comparing allelic frequencies in both controls and cases between regions we found no statistically significant differences (p = 0.098 and p = 0.595, respectively). Like the results observed when analyzing the total population, we found no differences in either the central or the northern region when comparing both allelic or genotypic frequencies between cases and controls (Table 2).

3.4. Stratified familial and sporadic PD allelic and genotypic frequencies

Lastly, we analyzed genotypic frequencies based on stratification by familial PD (n = 26) and sporadic PD (n = 82). No association for both familial PD (p = 0.48) or sporadic PD (p = 0.32) was observed (Table 3).

4. Discussion

4.1. Summary of previous studies

Although several studies have been carried out to evaluate the possible association between H1/H2 *MAPT* haplotype and PD, these have mainly been conducted in European populations. In this regard, in spite of the H1 haplotype being recognized as a risk factor for PD in caucasians [14], this association was not observed in German, Greek and Finnish populations [17,19], and thus suggests that it is ethnically dependent. With respect to Latin American populations, the only previous work was performed in population from the central region of Mexico, highlighting the need for more studies to determine the role of the H1/H2 haplotype in PD for these populations.

4.2. Regional genetic diversity in Mexican mestizo population and MAPT H1/H2

Mexican population, which is predominantly mestizo (composed of Amerindian, European, and, to a minor degree, African ancestries) has demonstrated regional genetic diversity that may affect biomedical traits in diseases [21,22]. Accordingly, our work included population from both the central and northern regions of the country. Although these genetic differences were reflected through a greater presence of H2 allele and H1/H2 genotype in cases and controls from the northern region compared to the central region, they were not statistically significant.

4.3. Multifactorial mechanisms for MAPT activation and PD?

We found no association between H1/H2 *MAPT* haplotype and PD risk, even after analyzing the population of each region independently. These results are consistent with those previously reported in Mexican mestizo population from the central region of Mexico [23] As PD is a multifactorial disease, perhaps our finding represents a distinct mechanism in the activation of *MAPT* in Mexican PD population; one that may very well be controlled by both genetic or epigenetic factors, including diet and environmental conditions. Future studies should consider additional analyses of current as well as new polymorphisms. Also, analysis of epigenetic changes of *MAPT* should be performed.

4.4. Study limitations

Finally, we would like to point out that our study has some limitations. We did not include a population from southern Mexico, which would allow a representation of the entire mestizo population of the country. Additionally, we did not determine the reported H1 subhaplotypes.

5. Conclusion

In conclusion, our results confirm no association between H1/H2 *MAPT* haplotype and PD in Mexican mestizo population and could serve as a useful reference when comparing among other ethnic groups in future studies.

Table 2
Allelic and genotypic frequencies of H1/H2 haplotype stratified by region and risk estimation to PD.

Region	Haplotype	Controls	Cases	*p* value	OR (95% CI)	*p* value
Central	H1	75 (0.96)	70 (0.90)	0.117*	1 (reference)	0.132
	H2	3 (0.04)	8 (0.10)		2.85 (0.72–11.20)	
	H1/H1	36 (0.92)	32 (0.82)	ND	1 (reference)	0.27
	H1/H2	3 (0.08)	6 (0.15)		2.25 (0.52–9.74)	
	H2/H2	0 (0)	1 (0.03)		ND	
Northern	H1	124 (0.90)	120 (0.87)	0.452*	1 (reference)	0.453
	H2	14 (0.10)	18 (0.13)		1.32 (0.63–2.79)	
	H1/H1	56 (0.81)	52 (0.75)	0.697*	1 (reference)	0.70
	H1/H2	12 (0.17)	16 (0.23)		1.44 (0.62–3.32)	
	H2/H2	1 (0.02)	1 (0.02)		2.26 (0.07–17.66)	

ND, not determined.
* Pearson's Chi-squared is significant at $p \leq 0.05$.

Table 3
Allelic and genotypic frequencies of H1/H2 haplotype stratified by familial and sporadic PD and risk estimation.

PD	Haplotype	Controls	Cases	*p* value	OR (95% CI)	*p* value
Familial	H1	47 (0.90)	45 (0.87)	0.539*	1 (reference)	0.541
	H2	5 (0.10)	7 (0.13)		0.68 (0.20–2.31)	
	H1/H1	21 (0.81)	20 (0.77)	ND	1 (reference)	0.48
	H1/H2	5 (0.19)	5 (0.19)		0.95 (0.21–4.36)	
	H2/H2	0 (.00)	1 (0.04)		ND	
Sporadic	H1	152 (0.93)	145 (0.88)	0.186*	1 (reference)	0.190
	H2	12 (0.07)	19 (0.12)		0.60 (0.28–1.28)	
	H1/H1	71 (0.87)	64 (0.78)	0.336*	1 (reference)	0.32
	H1/H2	10 (0.12)	17 (0.21)		0.52 (0.22–1.22)	
	H2/H2	1 (0.01)	1 (0.01)		0.88 (0.05–14.55)	

ND, not determined.
* Pearson's Chi-squared is significant at $p \leq 0.05$.

Contributions

EGM-M and JMS-P were involved in the experimental design and drafted the manuscript. OA-C and AS-C were involved in the experimental process and revised it critically for important intellectual content. EMM-H, FXC-J, and OL-L gave approval for the version to be published and were involved in revising it critically. LAR-C and GQ-C were involved in the clinical aspects of the study and revised the intellectual content of the manuscript.

Disclosure statement

The authors disclose no actual or potential conflicts of interest.

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Oxidative stress equilibrium during obstetric event in normal pregnancy

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Abstract

Objective: The aim of this study was to determine malondialdehyde (MDA) concentration as an oxidative stress marker and total antioxidant capacity (TAC) in pregnancy before and after perinatal event.
Methods: This study was performed on 200 healthy full-term pregnant women admitted to pregnancy resolution in Maternal-Child Hospital of Durango, Mexico. Oxidative stress and total antioxidant capacity were assessed through detection of lipid peroxidation by quantitation of thiobarbituric acid-reactive substances (TBARS) and total antioxidant capacity (TAC) through ferric reducing ability of the plasma (FRAP)
Results: Our results showed increased levels of MDA after vaginal delivery. Total antioxidant capacity was also increased after obstetric event, but it did not differ between vaginal delivery and caesarean section.
Conclusions: We demonstrated that MDA concentrations are increased two hours after obstetric event, and this increase correlates with VD. The TAC was increased as a compensatory mechanism during obstetric event. Another important finding is that women receiving analgesia administration in vaginal delivery, as well as dexamethasone administration in caesarean section, experienced a protector effect that decreased MDA levels.

Keywords: Delivery event, Oxidative stress, Malondialdehyde and Total antioxidant capacity.

Introduction

Oxidative stress represents a situation where there is an imbalance between the reactive oxygen species (ROS) and the availability and activity of antioxidants [1]. Due to the high potential to injure vital biological systems, ROS have been associated with more than one hundred diseases in their reproductive process. Several studies reported that parturition induces a strong oxidative stress response in mothers, implying an increased production of free radicals that must be controlled by their antioxidant system. This defence system can be overloaded during delivery in cases of abnormal oxygenation, where increased lipid peroxidation occurred [2]. In normal pregnancies, changes in oxygen concentrations are a very well-controlled phenomenon that has to provide a delicate balance between the metabolic needs of the placenta, foetus and mother, and the potential danger of ROS [3].

During the progression of normal labour, mothers experience a number of very stressful processes, such as pain, fear, anxiety and powerful contractions of the myometrium. The associated increase in intrauterine pressure and the degree of interruption or reduction in uteroplacental blood flow depend on the uterine contraction intensity [4], causing cycles of cellular hypoxia and reoxygenation; those are two essential elements of ischaemia–reperfusion injury [5].

The term oxidative stress has been defined as the “imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage [6]”. Importantly, it has been recently understood that ROS are the major signals involved in cardiovascular homeostasis [7].

Oxidative stress occurs in many pathological conditions and is now widely considered a major trigger of imbalance between protein synthesis and degradation [8]. Oxidative stress represents an inequity in oxidant and antioxidant levels. If there is an overproduction of oxidants that overwhelms antioxidant defences, oxidative damage of cells, tissues and organs ensues [9]. It has long been known that ROS are present in skeletal muscle [10] and can be generated during exercise and acute hypoxia periods [11]. As ROS can damage cell proteins, DNA and lipids through oxidation, they have been considered harmful agents. ROS production due to heavy exercise [12], as well as delivery process, have been shown to determine muscle damage, documented by increased lipid peroxidation. On the contrary, ROS production during moderate exercise causes positive adaptation, which is increased in insulin sensitivity, mitochondrial biogenesis and antioxidant defence systems [13]. When the production of ROS exceeds the capacity of antioxidant defence, oxidative stress has a harmful effect on the integrity of biological tissue through lipid peroxidation cascades or direct oxidation of membrane proteins. Malondialdehyde (MDA) is one of the small molecular weight pieces resulting from the fragmentation of polyunsaturated fatty acids undergoing attack by ROS and is generally accepted as an index of lipid peroxidation [14].

Material and Methods

Study population. This study was performed on 200 healthy full-term pregnant women admitted to pregnancy resolution in Maternal-Child Hospital of Durango, Mexico. The participants denied any chronic diseases, such as thyroid disease, liver disease, diabetes mellitus, hypertension or any pathological event during their pregnancy, including recent infections, premature membrane rupture and abnormal placentation. Written informed consent was obtained from all participants. The institutional Ethics Committee of Maternal-child Hospital approved the study. We obtained blood samples from the brachial vein (10 mL of blood) of each patient. The serum or plasma, obtained from centrifugation (3500 rpm for 10 min), was separated and distributed immediately in respective amounts necessary for marker determinations and stored at a temperature of -80°C, before carrying out the appropriate analysis.

Oxidative stress and total antioxidant capacity were assessed through detection of lipid peroxidation by quantitation of thiobarbituric acid-reactive substances (TBARS) and total antioxidant capacity (TAC) through ferric reducing ability of the plasma (FRAP).

TBARS assay. TBARS is a measurement of the reaction between MDA, a product of lipid peroxidation, and thiobarbituric acid (TBA) at temperatures of 90–100°C. The samples were assayed neat and in triplicate according to the method of Song et al. [15]. Briefly, 200 µL serum 0.2 mL of 8.1% sodium dodecyl sulphate, 1.5 mL of 20% acetic acid, 1.5 mL of 0.9% TBA and 0.6 mL of distilled water were added and vortexed. The reaction mixture was placed in a water bath at 95°C for 1 h. After cooling on ice, 1.0 mL of distilled water and 5.0 mL of butanol/pyridine mixture (15:1, v/v), were added and vortexed. After centrifugation at 10,000 rpm for 10 min, the pink MDA-TBA complex formed, and the resulting upper phase was determined at 532 nm. The concentration of TBARS was calculated using 1,5,3-tetraethoxypropane as a standard [15]. Results are expressed in nmol/L of MDA equivalents.

Total antioxidant capacity. The total antioxidant capacity was determined with the FRAP assay method, a simple test that measures the ferric reducing ability of plasma using 2,4,6 tripyridyl-s-triazine. Ferric to ferrous ion reduction at low pH causes a coloured ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions at a known concentration.

Statistical analysis

All tests were performed using IBM SPSS Version 21. Data were tested for normality distribution using the Kolmgorov–Smirnov test and appropriate distributional plots. Summary data are presented as mean ± standard error (SE) or median as appropriate for the distribution. Comparison concentrations of MDA and TAC between groups were made using the Student's t-test for independent groups. A p-value ≤0.05 was considered statistically significant.
The correlation tests were performed with Pearson's correlation and presented as p-value and ≤0.05

Results

Table 1 shows the socio-demographic and clinical characteristics of our study group. The mean age of women was 23.5 years, with a range of 14–43 years. The most frequent age range was 15–20 years (n=77, 38.5%). Thirty-three percent were primiparous, and 37% had three or more pregnancies. Socio-demographic differences between caesarean extraction (CE) and vaginal delivery (VD) groups were non-significant (Table 1).

Only 7% of women with VD requested epidural analgesia. In the case of CE, 83% received subarachnoid block, and 17% received epidural block. With respect to complications during delivery or surgical event, 95.5% of women did not present any complications, but 2% experienced obstetrical haemorrhage, 1.5% presented some type of dystocia event (shoulders or soft tissues) during delivery and 1% of these needed forceps.

All women received 10 UI of oxytocin in bolus immediately after VD or CE; women with CE (n=35) also received 20 mg of butilhioscina, 10 mg of metoclopramide, 60 mg of ketorolac and 8 mg of dexamethasone as surgical co-adjuvants. Women with obstetrical haemorrhage (n=4) received 800 µg of misoprostol, 0.2 mg of ergonovine maleate and 100 µg of carbetocin.

Hundred percent of newborns were alive, and 53.5% were female. The mean weight was 3.142 kg; 9.5% presented low birth weight, and 5% had macrosomy with respect to gestational age

Figure 1 showed that MDA concentrations were elevated after the obstetric event (2.43 ± 0.18 vs. 6.30 ± 0.37 nmol/L, p = 0.001). The comparison between VD and CE showed that MDA concentrations were significantly higher in VD group (6.6 ±5.5 vs 4.7 ±3.2, nmol/L p=0.001).

Plasma TAC concentrations were also elevated after the obstetric event (805.5 ±32.0 vs. 938.5 ±34.6 µmol/L, and a p-value=0.005) (Figure 2). In this case, the comparison between VD and CE showed that plasma TAC levels were slightly higher in the CE group (930.4±51.3 vs. 964±23.3 µmol/L) but without a statistic difference (p=0.616).

Discussion

The understanding of the role of free radicals in life sciences has consistently increased with time, and they have been connected to several physiological and pathological processes. ROS are generated from different exogenous and endogenous sources. The ROS produced in the tissues can inflict direct damage to macromolecules, such as lipids, nucleic acids and proteins. To counteract the harmful effects taking place in the cell, protection mechanisms have evolved to prevent and alleviate oxidative damages.

In pregnancy, many physiological changes occur in overall maternal systems, and the epidemiological characteristics can vary according to the geographical area. In our study, the women showed an average age of 23.5 years, similar to those reported nationally (24.2). However, our percentage of women aged between 14 and 20 (39.5%) years was higher than reported nationally (28.5%) [16]. These results show that our locality has a higher number of young pregnant women, a factor that has been associated with a more than doubled probability of maternal mortality by complications during pregnancy, intrapartum or postpartum period, in Mexico [17]. The delivery form in our study was mainly vaginal (83%), higher than reported by Heredia-Pi, L. et al. (53.1%) in Mexico [18]. With respect to BMI, we found that 34.5% of our study group was overweight or obese, similar to the results reported by Zonana-Nachach, A. et al. (38%) [19].

The increase in maternal lipid peroxidation and decrease of TAC is commonly associated with pathological conditions, such as preeclampsia or eclampsia [20]. On the other hand, the ROS increased slightly during normal pregnancy, suggesting it as a natural mechanism of uncomplicated pregnancies [21]. However, it is complicated to determine if this phenomenon is a cause or consequence of pregnancy.

In this study, the oxidative stress and total antioxidant capacity were assessed through detection of lipid peroxidation by quantitation of TBARS and TAC through FRAP two hours before and after perinatal event. We found a statistically significant difference in MDA levels ($p=0.001$) and plasma TAC concentrations ($p=0.001$) before versus after the perinatal event. Castro-Diaz, L. et al. [2] reported similar findings regarding oxidative stress status in pregnant women. They found an increase in plasma peroxides and erythrocyte membrane hydroperoxides, but a decrease in total antioxidant status during parturition, at three moments (at the beginning of the active phase of labour, at the start of expulsion and immediately after clamping cord). Induction of oxidative stress during periods of physical exhaustion, such as that experienced at the delivery moment, has been proposed as a cause of cellular membrane damage. This leads to an exacerbated inflammatory response and, therefore, pain and muscular fatigue [22]. Similar physiological processes that are present in women during pregnancy and postpartum independently of physical activities may be due to greater demand of oxygen [23]. In agreement with our results, we found an association between increased MDA levels in those women who had a VD measured two hours after obstetric event; these results differ from those reported by Hracsko, Z. et al. [24], who reported a higher concentration of MDA in women who had a pregnancy resolution by elective CE versus VD. However, it is important to note that Hracsko, Z. et al recollected blood samples at the time of delivery from umbilical cord. Our findings may be important because Arzalani-Zadeh, R. et al. [25] reported that MDA increased 24 h after surgical procedure. Similar results were reported by Zhang, G. L. et al. [26], who published that women under open surgical uterine myoma procedure had higher MDA values in serum versus the laparoscopic version of the same procedure, and that the levels of MDA in the women with open surgical procedure augmented at 24 h postsurgical procedure. So, we can say that MDA levels increase with time, and this fact may explicate our association with VD.

On the other hand, the negative association found between high MDA levels and dexamethasone administration in women whose pregnancy resolutions were by CE may be due to the anti-inflammatory effect of dexamethasone. This effect was demonstrated by Li, B. et al. [27] with an experiment in which rats with autoimmune encephalitis were administered dexamethasone, and the MDA levels decreased compared with a control group without disease and without dexamethasone. Another important association reported in this study is

the decrease of MDA levels in women who were administered analgesia during labour. This finding was also reported by Gyurkovits, Z. et al. [28], who compared the oxidative marker level in VDs with or without epidural analgesia. However, the impact of anaesthetics on oxidative stress is still not clear due to variations of patient's health conditions, types of surgery and quantities of anaesthetics.

Our findings showed increased TAC values after the obstetric event, independently of whether it was a VD or CE. However, Hracsko, Z. et al. also evaluated TAC with the same FRAP assay in pregnant women after delivery by VD or CE and found no differences between delivery forms, leading them to conclude that elective CE does not have an advantage over VD. The difference between our findings may be the moment the blood sample is collected. Additionally, the pregnant women were healthy, which may help to arrest the oxidative stress effect. The decrease in TAC status in women with pathological pregnancy, such as preeclampsia and gestational diabetes, is in agreement with results reported by Clerici, G. et al. [29]. Additionally, the statistically significant difference may be attributed to the effect of oxytocin administration in all women immediately after delivery and CE, as was reported by Akman, T. et al [30], who reported that administration of oxytocin significantly increased antioxidant capacity on rats.

Conclusion

Oxidative stress during the perinatal event can arise from overproduction of ROS by metabolic reactions that use oxygen and shift the balance between oxidant/antioxidant statuses in favour of the oxidants. However, diverse enzymatic mechanisms are also immediately activated to achieve an effective antioxidant response and offset ROS production to stop cellular damage and preserve maternal health. We demonstrated that MDA concentrations are increased two hours after obstetric event, and this increase correlates with VD. Another important finding is that pregnant women with analgesic administration during VD, or with dexamethasone administration in CE, experienced a protector effect that decreases MDA levels. On the other hand, the TAC was increased as a compensatory mechanism antagonist of ROS production and a protector system in human reproduction.

The birth of a new life carries in the mother generations of ROS molecules. However, it is surprising how maternal physiology produces compensatory antioxidant mechanisms for maintaining health and preserving reproductive function.

Finally, we consider it important to continue studying the oxidative/antioxidant status in pregnant women and their chosen methods of obstetric resolution. Greater understanding is necessary to determine the risk of development of chronic cardiovascular diseases and pathologies related to oxidative/antioxidant equilibrium.

Declaration of interest

The authors report no declarations of interest. This work was supported by Servicios de Salud de Durango and Universidad Juárez del Estado de Durango.

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Figure 1. MDA concentration 2 h before and after obstetric event.

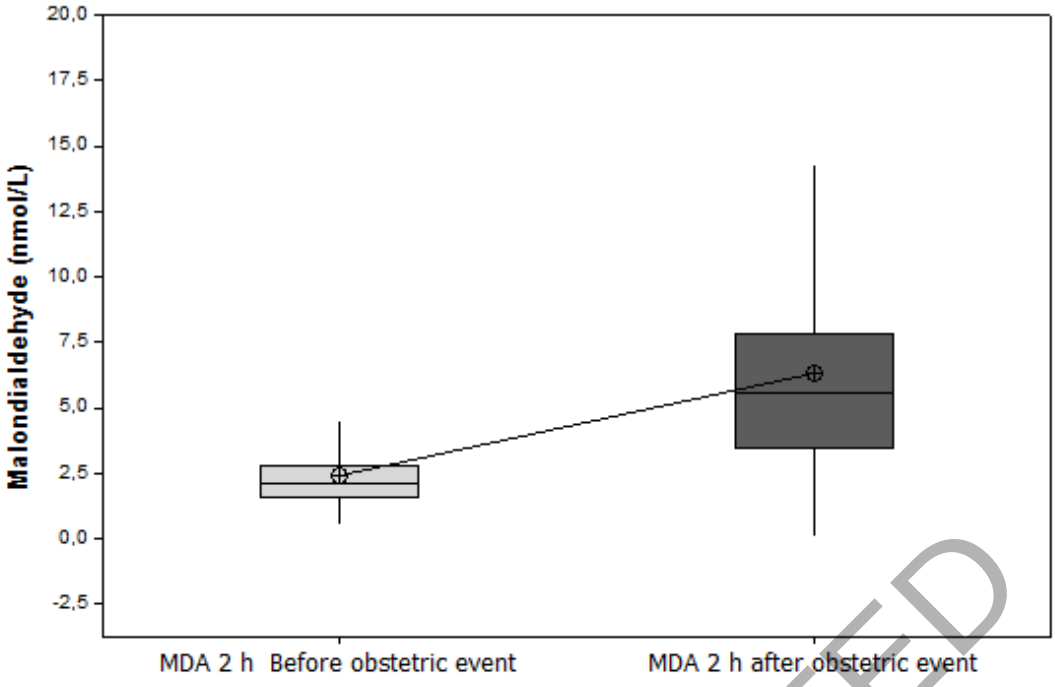

Figure 2. Plasma TAC concentration 2 h before and after obstetric event.

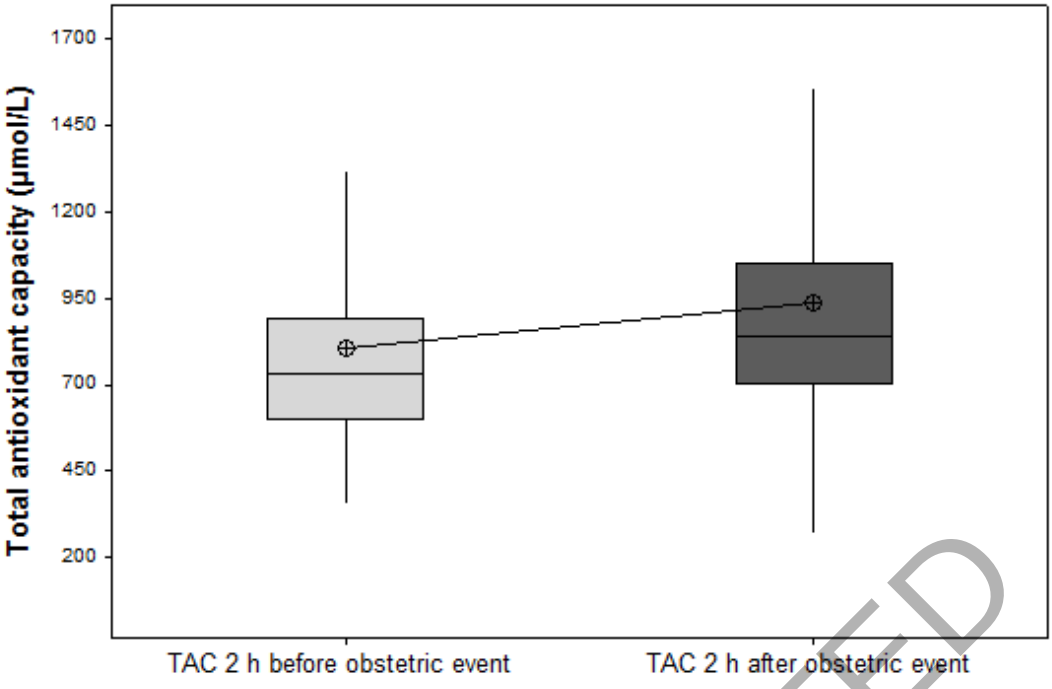

Table 1. Clinical and socio-demographic characteristics in pregnant women

Characteristic	Total (n=200)	Vaginal delivery (n=165)	Caesarean extraction (n=35)	p-value *
Weight (Kg)	60.2±12.3	59.2±11.8	65±13.7	0.327
Body mass index	23.5±4.5	23.2±4.4	25.1±4.8	0.688
Age (years)	23.6±6.1	23.5±6.3	24.1±5.4	0.480
Gestation age (weeks)	39.2±1.1	39.1±1.1	39.3±1.1	0.991
Newborn weight (kg)	3.14±0.4	3.1±0.41	3.29±0.35	0.181
Pregnancies number	2.34±1.3	2.39±1.4	2.09±1.0	0.072

* Student t-test
p≤0.05 is statistically significant

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RESEARCH ARTICLE Open Access

Arsenic exposure and risk of preeclampsia in a Mexican mestizo population

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Abstract

Background: Exposure to arsenic in drinking water has been associated with various complications of pregnancy including fetal loss, low birth weight, anemia, gestational diabetes and spontaneous abortion. However, to date, there are no studies evaluating its possible association with preeclampsia.

Methods: This case–control study involved 104 preeclamptic and 202 healthy pregnant women. The concentrations of arsenic in drinking water and urine were measured using a Microwave Plasma-Atomic Emission Spectrometer.

Results: We found relatively low levels of arsenic in household tap water (range of 2.48–76.02 μg/L) and in the urine of the participants (7.1 μg/L vs 6.78 μg/L in cases and controls, respectively).

Conclusions: The analysis between groups showed for the first time that at these lower levels of exposure there is no association with preeclampsia.

Keywords: Preeclampsia, Arsenic, Drinking water

Background

Preeclampsia (PE) is a disorder peculiar to pregnancy and a major cause of maternal death and adverse fetal outcome [1]. In developing countries where access to health care is limited, PE is a leading cause of maternal mortality, with estimates of more than 60,000 maternal deaths per year [2] Although the exact pathophysiologic mechanisms of PE remain elusive, studies to date have implicated multiple processes, including the following: abnormal trophoblastic invasion, vasospasm, platelet activation, imbalance in the vasomotor-regulating factors and placental ischemia [3]. PE is characterized by increased oxidative stress due to the imbalance between lipid peroxidation and antioxidant defense mechanisms, leading to endothelial dysfunction and free radical mediated cell injury [4].

Arsenic-contaminated drinking water represents a major public health problem internationally [5–8]. The World Health Organization (WHO) and U.S. Environmental Protection Agency (EPA) standard for arsenic level in drinking water is 10 μg/L [9, 10]. Arsenic (As) is an established carcinogen and is also associated with a wide range of other chronic illnesses, such as diabetes, hypertension, and vascular diseases [11].

Oxidative stress has been identified as an important mechanism of As toxicity and carcinogenicity. In particular, As induces oxidative DNA damage and lipid peroxidation [12–16]. Oxidative stress and disrupted antioxidant systems have been shown to be involved in a wide range of pregnancy complications such as impaired fetal growth, PE, and miscarriage [17, 18].

Besides the generation of oxidative stress as a possible mechanism by which As may be associated with PE, Shin Le et al. reported that exposure to environmentally relevant concentrations of As (2.5 μM of AsNaO2) inhibit the migration of EVT cells (a human extravillous trophoblast cell line) in vitro, therefore, a similar mechanism may be occurring in vivo [19].

Several studies have been conducted to determine the association between chronic As exposure and adverse pregnancy outcome. Excess spontaneous abortion,

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stillbirth, and preterm birth rates among women with chronic As exposure have been reported [20–23]. However, to date there are no reports that show an association between As exposure and PE. This study evaluates whether As exposure from drinking water is associated with PE in a population of northern Mexico.

Methods

Patient recruitment

This prospective case–control study was approved by the Research Ethics Committee of the General Hospital of the Ministry of Health of Durango, Mexico in accordance with the Code of Ethics of the Declaration of Helsinki. Signed informed consent was obtained from all patients and controls before participation in the study. The sample size was calculated using the formula $n = (Z_{\alpha/2} + Z_{\beta})^2\ \dot{p}\ (1-\dot{p})\ (r+1)/d^2r$. The n needed to achieve 80 % power with an alpha of 0.05 was 94 (cases) and 188 (controls). Finally, we recruited 104 women diagnosed with PE (cases) and 202 healthy pregnant women (controls). The inclusion criteria were all those women diagnosed with mild PE (blood pressure (BP) ≥ 140/90 mmHg and proteinuria ≥ 30 mg/dL), severe PE (BP ≥ 160/110 mmHg and proteinuria ≥ 2000 mg/dL) and eclampsia (defined as occurrence, in a woman with PE, of seizures that cannot be attributed to other causes). The control group was conformed by healthy pregnant women attending the same hospital; without hypertensive, pathological or metabolic disorders during pregnancy. Follow up was given to the control group to corroborate the normality of the blood pressure values.

Sample collection

Within 1–3 weeks of delivery, a drinking water sample was collected at the homes of each of the study participants. Drinking-water samples were collected based on the subject's primary drinking water source. Maternal spot urine samples were collected at the hospital before delivery and immediately transported to the laboratory. Samples were stored at −80 °C until processing.

Detection of As in drinking water and urine

The concentrations of As in drinking water (DW) and urine were measured in the toxicology laboratory of Scientific Research Institute of the Universidad Juárez del Estado de Durango (UJED) using a Microwave Plasma-Atomic Emission Spectrometer (MP-AES 4100). The Trace Elements in Water standard reference material (SRM 1643e) (National Institute of Standards and Technology, Gaithersburg, MD) was used for quality control. The limit of detection for As in DW by MP-AES was 0.5 μg As/L. For urine analysis, six point calibration curves were prepared. To compensate for variation in the dilution of the urine (caused by variation in fluid intake, time of sampling, temperature, and physical activity), we adjusted the concentrations by specific gravity.

Statistical analysis

Independent sample Student's *t*-tests were performed using SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA). Odds ratios (ORs) as estimates of relative risk of the disease were calculated with 95 % confidence intervals (95 % CIs). The ORs were adjusted for variations in age and weeks of pregnancy by means of a multivariate logistic regression model. Mann–Whitney *U* test was used when the data were not normally distributed. For analysis, our patients were stratified into 3 groups based on As levels in DW (Table 3). The Group 1 (G1) presented levels lower than 10 μg/L, group 2 (G2) levels between 10.1 μg/L and 25 μg/L and group 3 (G3) levels above 25.1 μg/L

Results

Clinical characteristics for controls and cases are shown in Table 1. Of the 104 women diagnosed with PE, 13 had mild PE, 72 severe PE and 19 eclampsia. Variables that showed a difference between groups were family history of PE, systolic and diastolic blood pressure (mm Hg), weeks of pregnancy and body mass index (Table 1). The range of As concentration in household tap water was 2.48–76.02 μg/L with more than 95 % of the participants having As levels higher than 10 μg/L. The mean concentration of As in DW was 39.58 μg/L and 40.49 μg/L for cases and controls, respectively; there were no statistically significant differences (Table 2, $p = 0.816$). While the WHO sets a maximum concentration of 10 μg/L in DW, the authorities in Mexico have set a maximum concentration of 25 μg/L (NOM-127-SSA1-1994) [24]. For this reason, the OR was estimated stratifying our patients into 3 groups based on As levels in DW. The results of Table 3 show that although the group exposed to concentrations above 25 μg/L presents an increased risk (OR = 1,715). This difference is not statistically significant ($p = 0.214$).

Table 1 Clinical characteristics for cases and controls

Clinical features	Controls (*n = 202*)	Cases (*n = 104*)	P-*value*
Age (years)	24.30 (7.078)[a]	24.39 (7.349)[a]	.92[b]
Weeks of pregnancy	37.49 (3.96)[a]	35.82 (3.97)[a]	0.001[b]
Systolic BP (mm Hg)	111.74 (10.82)[a]	158.36 (16.41)[a]	<0.0001[b]
Diastolic BP (mm Hg)	70.39 (9.97)[a]	101.21 (10.3)[a]	<0.0001[b]
Number of pregnancies	2.26 (1.40)[a]	2.34 (2.49)[a]	0.718[b]
Body mass index	24.61 (5.22)[a]	27.63 (5.82)[a]	<0.0001[b]
PE antecedent	13/202	14/104	0.045[c]

[a]Mean ± Standard deviation
[b]Independent sample *T* test
[c]Chi square test

Table 2 Water and urine arsenic levels in cases and controls

Arsenic μg/L	Controls (*n = 202*)	Cases (*n = 104*)		P-*value*
Water	40.49 (16.40)[a]	39.58 (26.43)[a]		0.816[b]
Urine	6.78 (3.48)[a]	7.1 (5.74)[a]		0.428[c]
		Mild PE *n* = 13	Severe PE/eclampsia *n* = 91	P-*value*
Water		46.03 (20.65)[a]	38.62 (26.87)[a]	0.519[b]
Urine		7.82 (6.87)[a]	7.03 (5.67)[a]	0.788[c]

[a] Mean ± Standard deviation
[b] Independent sample *T* test
[c] Mann–Whitney *U* test

Total urinary As concentration (U-tAs) was also evaluated. The mean concentration of U-tAs was 7.1 μg/L and 6.78 μg/L for cases and controls, respectively; there were no statistically significant differences (Table 2, $p = 0.428$). With the intention to establish whether As may be associated with the severity of PE, the cases were stratified in mild PE and severe PE/eclampsia. The results of Table 2 show that there is no statistically significant differences in the U-tAs (p =0.788). The risk of PE by U-tAs was estimated piling up to the patients in tertiles. The results in Table 3 show that at these levels, U-tAs is not a risk for PE.

Finally, we evaluated the correlation between As in DW and U-tAs. We observed an increase in the U-tAs associated with higher levels of As in DW. G1 presented a mean of 3.39 μg/L, G2 of 6.67 μg/L and G3 of 7.8 μg/L. However, the correlation coefficient was very low ($R^2 = 0.036$).

Discussion

To our knowledge this is the first study that evaluates if As exposure from DW is associated with PE. The As concentrations in household tap water (2.48–76.02 μg/L) were consistent with those previously found by our working group in the wells that provide DW to the city of Durango [25, 26]. Although these concentrations are not as high as those reported in other countries [27–30] or even in other regions of our own locality [31], there is a tremendous interest in the evaluation of regions with low or moderate As exposure in accordance with the increasingly clear evidence that relatively low levels of As can have health effects. Our comparative analysis between controls and cases evidenced no statistically significant differences. In addition, no differences were found in the analysis based on the severity of the PE.

The analysis of U-tAs showed a mean of 7.1 μg/L for cases and 6.78 μg/L for controls. These U-tAs levels are clearly lower than those reported among pregnant women in Bangladesh (80 μg/L) [32] and even lower than those reported in pregnant women in the nearby region known as Comarca Lagunera (23.3 μg/L) [33]. In our study we didn't find an association between U-tAs and PE or an association with the severity of PE. Recently, Joy-Mendez et al. found no association between serum As levels and blood pressure in a cohort of pregnant women from Mexico city [34]. They reported a mean of 15.2 μg/L of As in serum. Although they don't evaluate PE, our results can be considered similar.

In contrast to our results, several reports have associated As exposure with pregnancy complications including low weight of the newborn [35], fetal death [36], gestational diabetes [32], anemia [37] and spontaneous abortions [38], however, these associations appear at significantly higher levels of As (e. g., fetal death, U-tAs >200 μg/L or spontaneous abortions, As in DW >100 μg/L).

Our results could be interpreted on the one hand, as a confirmation of no association between As and PE, at least at these low levels. On the other hand, they might suggest that we need higher levels of As exposure to be able to observe the association.

Our study has some limitations. Although the participants state that their main source of water is from the tap, we can't rule out that As can come from other sources of drinking water (e.g., bottled water), some

Table 3 Odds ratio estimation by ranges of arsenic in water and urine

Water arsenic	OR* (95 % CI)	P-*value*	Urine arsenic	OR* (95 % CI)	P-*value*
Group 1[a] *n = 10*	Reference		Tertile 1[d] *n = 102*	Reference	
Group 2[b] *n = 69*	1.486 (0.200–11.025)	0.698	Tertile 2[e] *n = 102*	1.400 (0.748–2.621)	0.698
Group 3[c] *n = 227*	1.715 (0.732–4.019)	0.214	Tertile 3[f] *n = 102*	0.788 (0.411–1.512)	0.214

[a] DW As < 10 μg/L
[b] DW As 10.1–25 μg/L
[c] DW As >25 μg/L
[d] U-tAs ≤7.4956 μg/L
[e] U-tAs >7.4956 ≤ 11.4911 μg/L
[f] U-tAs >11.4911 μg/L
* ORs were adjusted for age and weeks of pregnancy

food, or by some occupational exposure. Another limitation is that we didn't find high levels of U-tAs, so we can't establish in our study if higher levels of urinary As are or are not associated with PE.

The evaluation of pregnant women with higher levels of As as well as the analysis of other factors (e.g., genetic or nutritional) becomes necessary to confirm and strengthen our findings.

Conclusions

First, it is shown that the majority of our population is exposed to As levels higher than that established by the WHO. In addition, our work suggests for the first time that there is no association between As exposure and PE.

Abbreviations

As, arsenic; DW, drinking water; EPA, environmental protection agency; ORs, odds ratios; PE, preeclampsia; UJED, Universidad Juárez del Estado de Durango; U-tAs, urinary arsenic concentration; WHO, World Health Organization

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Availability of data and materials

The data will not be shared in order to protect the participants' anonymity.

Authors' contributions

EMMH, ATV and OLL carried out the statistical analysis and helped to draft the manuscript. EIAS, SMSP, FVA and MBS carried out the integration of groups and sampling of household tap water. FXCJ and MAD carried out the arsenic determinations. JMSP and ASC conceived of the study, and participated in its design and coordination and drafted the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of the General Hospital of the Ministry of Health of Durango, Mexico. Informed signed consent was obtained from study participants.

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RESEARCH ARTICLE

TNF-α Polymorphisms and Maternal Depression in a Mexican Mestizo Population

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Abstract: ***Background***: Depressive disorders are common during pregnancy. There is compelling evidence that the inflammatory response system is important in the pathophysiology of depression. Higher concentrations of proinflammatory cytokines including tumor necrosis factor-alpha (TNF-α) in depressed subjects have been described. Because several polymorphisms in the TNF-α promoter region are known to affect its gene expression, the aim of this study was determine whether TNF-α -857C/T, -308G/A, and -238G/A polymorphisms confer susceptibility to depression during pregnancy in a Mexican mestizo population.

Methods: This case-control study involved 153 depressed pregnant women and 177 controls. Polymorphisms were genotyped using real-time PCR. Odds ratios (OR) and 95% confidence intervals adjusted by age, body mass index, number of pregnancies, months of pregnancy and number of abortions were used to estimate risk.

Results: The -857CT genotype was found to increase the risk for depression (OR= 1.73, 95% CI= 1.06-2.82). In contrast, the -238GA genotype reduced the risk (OR= 0.33, 95% CI= 0.14-0.72). The -308G/A polymorphism was not associated with risk for depression. Finally, the C857-G308-A238 haplotype was associated with a decreased risk of depression (OR= 0.35, 95% CI= 0.15-0.82).

Conclusion: Our results show for the first time an association between *TNF-α* -857C/T and -238G/A polymorphisms and prenatal depression in Mexican mestizo population.

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1. INTRODUCTION

Depression during pregnancy can lead to behavioral changes such as the abandonment of prenatal controls, poor adherence to medical indications, consumption of tobacco, drugs, and alcohol with potentially devastating consequences for both mother and baby. Almost 10% of pregnant women are diagnosed with depression. This percentage may be increased depending on cultural and socioeconomic conditions [1]. A number of factors have been associated with depression during pregnancy: lack of family or social support, stressful life events, tobacco use, hormonal changes, anxious temperament, history of mental illness and genetic predisposition, are some examples [2].

Besides this, several reports suggest that depression is an inflammatory disorder mediated by proinflammatory cytokines, such as interleukins 2, -6, and -12 and tumor necrosis-alpha (TNF-α) [3-5].

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TNF-α is a proinflammatory cytokine that strongly contributes to inflammatory and immune responses by inducing a cascade of various inflammatory cytokines; it is produced by monocytes, macrophages and T and B lymphocytes, and also by microglia in the central nervous system [6].

The role of TNF-α in depression has been evaluated in both epidemiological studies and animal models. A meta-analysis showed that TNF-α is commonly elevated in depressed patients [7]; also, the administration of TNF-α to rats induces a spectrum of behavioral changes including social withdrawal, decreased motor activity, reduced food intake and sleep alterations [8].

The TNF-α gene is located on chromosome 6p21.3, within the class III region of MHC [9, 10]. Single nucleotide polymorphisms (SNPs) in the TNF-α gene, including -238G/A (rs361525), -308G/A (rs1800629), and -857C/T (rs1799724) have been described [11]. Because these SNPs in the TNF-α promoter region have been associated with different TNF-α expression profiles and circulating TNF-α levels, they can modulate inflammatory processes, disease development and response to treatment [12]. For that reason, the main goal of our study determined if -238G/A, -308G/A and -857C/T polymorphisms of TNF-α gene confer susceptibility to depression during the prenatal period in a Mexican mestizo population.

2. MATERIALS AND METHOD

2.1. Patient Recruitment

Blood samples were obtained from patients of a previous study conducted at General Hospital of the Secretary of Health in Durango City from March 2015 to February 2016 [13].

2.2. DNA Extraction and Genotyping of Samples

The DNA extraction from blood samples was performed using the QIAamp DNA blood extraction kit (Qiagen, Hilden, Germany). The genotypes were assessed using TaqMan assays (Applied Biosystems) as described previously [14]. The predesigned assays were C-11918223-10, C-7514879-10, and C-2215707-10 (-857C/T, -308G/A, and -238G/A, respectively.

2.3. Statistical Analysis

The clinical characteristics were expressed as mean and were compared using the Student's *t*-test. The allele and genotype frequencies were calculated by direct counting. Deviation from the Hardy-Weinberg equilibrium (HWE) constant was tested using a χ^2 test with 1 degree of freedom. The differences of distributions of the polymorphisms were performed by χ^2 analysis using SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA); $p < 0.05$ was considered statistically significant. The odds ratio was calculated from allelic and genotype frequencies with 95% confidence intervals (95% CI) using the SNPstats software program (Catalan Institute of Oncology).

3. RESULTS

A total of 330 pregnant women were enrolled in the study (153 depressed pregnant women and 177 controls). Of the 153 women diagnosed with depression, 93 had mild depression and 60 severe depression. Only the body mass index showed a difference between groups ($p = 0.036$, Table **1**).

Allelic and genotypic frequencies of -857C/T, -308G/A, and -238G/A TNF-α polymorphisms are shown in Table **2**. All polymorphisms were in HWE. The allelic frequencies of -857C/T and -238G/A showed statistically significant differences between groups ($p= 0.030$ and $p= 0.0019$, respectively). Also, these differences were observed in the genotypic frequencies of -857C/T polymorphism ($p = 0.047$). No differences in allelic or genotypic frequencies between cases and controls were observed in -308G/A polymorphism (Table **2**, $p > 0.05$).

The risk of depression by the presence of these polymorphisms was determined. A logistic regression model adjusted for age, body mass index, number of pregnancies, months of pregnancy and number of abortions was used. The results of Table **3** showed that the -857CT genotype is a risk factor (OR= 1.73, 95% CI= 1.06-2.82) and that the -238GA genotype is a protector factor (OR= 0.33, 95% CI= 0.14-0.72) for depression in pregnant women. Furthermore, a significant trend was observed for both polymorphisms (p for trend = 0.035 and 0.001). Finally, haplotype analysis showed that the C857-G308-A238 haplotype was significantly associated

Table 1. Clinical characteristics of depressed (cases) and healthy pregnant women (controls).

Clinical Features	Cases, *n* = 153	Controls, *n* = 177	*p*-value
Age (years) [a]	23.49 (8.72)	23.58 (8.05)	0.925 [b]
BMI [a]	27.99 (5.71)	26.71 (5.32)	0.036 [b]
Number of pregnancies [a]	2.21 (1.52)	2.12 (1.51)	0.606 [b]
Months of pregnancy [a]	6.54 (1.52)	6.82 (1.42)	0.086 [b]
Number of abortions [a]	0.13(0.38)	0.17 (0.49)	0.439 [b]

[a]Mean (± Standard deviation).

[b]Independent sample *T* test.

Table 2. Allele and genotype frequencies of TNF-α polymorphisms in depressed pregnant women (cases) and healthy pregnant women (controls).

		Cases *n* = 153	Controls *n* = 177	*p*-value
-857C/T	C	0.77	0.84	0.030 [a]
	T	0.23	0.16	
	C/C	0.59	0.72	0.047 [a]
	C/T	0.37	0.25	
	T/T	0.04	0.03	
-308G/A	G	0.94	0.94	0.929 [a]
	A	0.06	0.06	
	G/G	0.89	0.90	0.175 [a]
	G/A	0.11	0.08	
	A/A	0	0.02	
-238G/A	G	0.97	0.92	0.0019 [a]
	A	0.03	0.08	
	G/G	0.95	0.84	ND [a]
	G/A	0.05	0.16	
	A/A	0	0	

[a] Pearson's Chi-squared is significant at $p \leq 0.05$.

ND, not determined.

Table 3. TNF-α polymorphisms association with depression in pregnant women.

		Cases n	Controls n	OR	95% CI	p-value
-857C/T	C/C	90	126	1.00	(referent)	0.078
	C/T	57	45	1.73	(1.06-2.82)	
	T/T	6	6	1.54	0.44-5.40	
	p value for trend					0.035
-308G/A	G/G	136	160	1.00	(referent)	0.14
	G/A	17	14	1.42	0.66-3.05	
	A/A	0	3	ND	ND	
	p value for trend					0.960
-238G/A	G/G	145	148	1.00	(referent)	0.0035
	G/A	8	29	0.33	0.14-0.72	
	A/A	0	0	ND	ND	
	p value for trend					0.001

ND, not determined.

with a decreased risk of depression (OR= 0.35, 95% CI= 0.15-0.82, Table **4**).

4. DISCUSSION

The continued search for risk markers in depressed pregnant women remains of great interest because of the wide range of negative outcomes such as social isolation [15], marital discord [16], child delays in motor or intellectual development [17], restricted fetal growth and elevated stress reactivity in infants [18, 19], among others.

Peripheral inflammation can lead to depression through several immune-mediated pathways that transmit the signal from the periphery to the central nervous system. Patients with major depressive disorder exhibit all of the cardinal features of an inflammatory response, including increased

Table 4. Frequencies and association of TNF-α (−857C/T, −308G/A and -238G/A) haplotypes with depression in pregnant women.

Haplotypes	Cases	Controls	OR	95% CI	*p*-value
C857-G308-G238	0.69	0.70	1.00	(referent)	
T857-G308-G238	0.225	0.161	1.39	(0.91 - 2.12)	0.12
C857-A308-G238	0.054	0.056	1.03	(0.53 - 2.00)	0.93
C857-G308-A238	0.024	0.081	0.35	(0.15 - 0.82)	0.016
C857-A308-A238	0.001	0	ND	ND	ND

ND, not determined.

expression of pro-inflammatory cytokines and their receptors and increased levels of acute-phase reactants, chemokines and soluble adhesion molecules in peripheral blood and cerebrospinal fluid [20]. In this context, SNPs that modulate the expression of TNF-α or any other pro-inflammatory cytokine may have a potential role in susceptibility to depression.

The polymorphisms evaluated in this work have previously been associated with differences in TNF-α gene expression. Furthermore, they also have been associated with some disorders including attempt suicide [21], schizophrenia [22], obsessive-compulsive disorder [23], major depressive disorder [24] and post-stroke depression [25]. However, there are no studies evaluating their possible role in prenatal depression.

Our results showed that the -857CT and -238GA genotypes increase and reduce the risk to develop depression in our population, respectively. These results are consistent with evidence suggesting an increase in proinflammatory cytokines in depressed patients. The TNF-α-857T allele was reported to be associated with high TNF-α production in *in vitro* cell proliferation studies [26]. Also, the T allele was associated with increased transcription of TNF-α in a Chinese population and high serum levels of TNF-α in the Indian and Japanese population [12, 27-29]. On the other hand, the −238A allele was reported to down-regulate TNF-α expression [30, 31]. Moreover, the −238G allele was related to high TNF-α mRNA expression and high serum TNF-α concentrations in rheumatoid arthritis and in knee osteoarthritis patients [32, 33].

Concerning -308G/A SNP, the literature is controversial. Studies with both increased [34-36] and decreased [37-40] TNF-α plasma or mRNA levels associated with the −308A allele have been published. Also, some works suggest an association of this SNP with depression [24, 25] but others not [41, 42]. Our results suggest no association between the -308G/A SNP and prenatal depression.

In relation to the genotypic frequencies, previous studies reported a frequency of 0.746 CC, 0.248 CT and 0.004 TT for -857CT SNP, 0.93 GG and 0.07 GA for -308G/A SNP and 0.89 G/G and 0.11 G/A for -238G/A SNP [32, 43]. We found very similar results for our group of controls.

Interestingly, the C857-G308-A238 haplotype was associated with a decreased risk of depression. As already mentioned, we would expect higher levels of proinflammatory cytokines in patients with depression. Therefore, our results suggest that the presence of the C857 allele (associated with decreased transcription of TNF-α) would have a greater effect than that of the C857 allele (associated with increased transcription of TNF-α) and consequently, lower amounts of TNF-α protein would be produced in those individuals who have the C857-G308-A238 haplotype. However, further experimentation will be needed to prove it.

There are some limitations in our study. First, the effect of SNPs on TNF-α gene expression or circulating TNF-α levels was not evaluated. These data would be of great interest, in particular regarding the controversial -308G/A SNP findings in previous reports. Second, only pregnant women from the northern region of México were included. To establish these polymorphisms as risk markers in the general Mexican population, it will be necessary to carry out additional studies that include women from all regions of the country, based on the demonstrated genetic differences between subpopulations from different regions throughout México [44].

CONCLUSION

Our results show for the first time an association between TNF-α -857C/T and -238G/A polymorphisms and prenatal depression in a Mexican mestizo population.

LIST OF ABBREVIATIONS

HWE = Hardy-Weinberg Equilibrium

SNP = Single Nucleotide Polymorphism

TNF-α = Tumor Necrosis Factor-alpha

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Ethics Committee of the General Hospital of the Secretary of Health in Durango City, Mexico approved this study, and written informed consents were obtained from all participants and from the next of kin of minor participants.

HUMAN AND ANIMAL RIGHTS

The study was conducted in accordance with the Helsinki Declaration.

CONSENT FOR PUBLICATION

Written informed consents were obtained from all participants and from the next of kin of minor participants.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Article

Novel Mixed-Type Inhibitors of Protein Tyrosine Phosphatase 1B. Kinetic and Computational Studies

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Abstract: The Atlas of Diabetes reports 415 million diabetics in the world, a number that has surpassed in half the expected time the twenty year projection. Type 2 diabetes is the most frequent form of the disease; it is characterized by a defect in the secretion of insulin and a resistance in its target organs. In the search for new antidiabetic drugs, one of the principal strategies consists in promoting the action of insulin. In this sense, attention has been centered in the protein tyrosine phosphatase 1B (PTP1B), a protein whose overexpression or increase of its activity has been related in many studies with insulin resistance. In the present work, a chemical library of 250 compounds was evaluated to determine their inhibition capability on the protein PTP1B. Ten molecules inhibited over the 50% of the activity of the PTP1B, the three most potent molecules were selected for its characterization, reporting Ki values of 5.2, 4.2 and 41.3 μM, for compounds **1**, **2**, and **3**, respectively. Docking and molecular dynamics studies revealed that the three inhibitors made interactions with residues at the secondary binding site to phosphate, exclusive for PTP1B. The data reported here support these compounds as hits for the design more potent and selective inhibitors against PTP1B in the search of new antidiabetic treatment.

Keywords: protein tyrosine phosphatase 1B; type 2 diabetes; benzimidazole derivatives; enzyme inhibition; docking; molecular dynamics

1. Introduction

In its sixth edition, the Atlas of Diabetes reported 415 million diabetics worldwide, surpassing in half the time the twenty year projection [1,2]. Type 2 diabetes is the most frequent type of the disease; it is characterized by a defect in the secretion of insulin and resistance in its target organs. For its treatment there are several hypoglycemic agents, these have different mechanisms of action such as an increase in insulin production, decrease of the hepatic glucose production, limiting the

absorption of postprandial glucose, and inhibiting gluconeogenesis [3]. Nevertheless, after 3 or 4 years of treatment the efficacy of these drugs is diminished, even with combinations among them, and insulin administration becomes necessary [4]. Therefore, there is an urgency for new drugs with other mechanisms of action that can provide different alternative treatments for type 2 diabetes.

To this end, one of the main strategies consists in promoting the action of insulin [5], and the attention has been focused in the protein tyrosine phosphatase 1B (PTP1B), a protein which overexpression and increase in its activity has been related in many studies with insulin resistance [6–8]. PTP1B works specifically by dephosphorylating residues of phosphotyrosine both from the insulin receptor (IR) and insulin receptor substrate (IRS) [9]. In a recent study, Munc18c was discovered as a substrate for the PTP1B, it is related to the regulation of glucose transporter GLUT4 in adipocytes, allowing or impeding, the insertion of the vesicle into the membrane [10]. These and other evidences [11–13] validate this enzyme as a potential therapeutic target against type 2 diabetes.

Since the establishment of the PTP1B as a biological target, there has been an effort to obtain inhibitors of its activity [14,15]. Different strategies such as diverse computational techniques, natural products research, and medicinal chemistry have been applied in the search for PTP1B inhibitors [16–20]. The first efforts to obtain inhibitors consisted in the search for phosphotyrosine (pTyr) mimetics such as difluoromethylene phosphate (DFMP) [21–24], carboxylic acids [25–28], 1,2,5-thiazolidin-3-one 1,1-dioxide (TZD), and the (*S*)-isomer of isothiazolidinone ((*S*)-IZD) [24,29], which achieve different degrees of inhibition of the enzyme. Furthermore, natural products and some derivatives have been reported too [30,31], as well as small molecules optimized from the previous ones [23,32]. Nevertheless, finding a potent, selective and with good oral availability molecule is still a challenge to be overcome.

The highly conserved catalytic site in the phosphatases family hinders the finding of a selective molecule, especially against its closest homologous T-cell protein tyrosine phosphatase (TCPTP) [33,34]. One strategy is to seek interactions with specific sites of the PTP1B [35–37]. In this sense, the enzyme has two aryl phosphate binding sites, a catalytic site with high affinity that contains the Cys215, and another one with low affinity that contains the Arg24 and Arg254. The former, denominated as site B, is specific for PTP1B. A few molecules based on this concept have reached clinical trials, among which ertiprotafib, ISIS 113715 and trodusquemine may be highlighted [38–40], however they did not continue to later stages.

In the present work, a chemical library composed by 250 compounds was evaluated to determine their inhibition capabilities on the PTP1B. The three most potent molecules were selected for further characterization, including their mechanism and inhibition constants. Structural studies of the PTP1B-inhibitor interaction were performed through docking and molecular dynamics simulations as well as an estimation of their drug-like and toxicological properties.

2. Results and Discussion

2.1. Compounds Screening

With the aim of finding new compounds capable of inhibiting PTP1B activity, a chemical library of 250 small molecules was evaluated at 200 μM. From the total of compounds evaluated, 26 inhibited the enzyme activity by more than 60%, 26 of them between 31–59% (Table S1), and the rest below 30%. The characteristics of the ten most potent compounds are shown in Table 1, and their structures in Figure 1.

According to their structure, the three most potent PTP1B inhibitors (compounds **1**, **2** and **3**) all contain a benzimidazole nucleus. The analysis suggests that bulky substituents are required at positions 2 (**1** vs. **9** and **10**) and 5 (**1** vs. **10**) of the benzimidazole skeleton to increase their inhibition capability. The presence of the benzimidazole nucleus in the structure of the PTP1B inhibitors has been reported before, nevertheless, due to their substituents, they showed permeability and specificity problems with respect to other phosphatases [41–43].

Table 1. The ten most potent PTP1B inhibitors.

Molecule	MW [a]	HBD [a]	HBA [a]	LogP [a]	Drug Likeness [b]	Binding Energy (Kcal/mol)	% Inhibition (200 μM)
1	567.27	2	7	6.68	1.07	−4.20	100
2	466.96	2	6	6.00	0.86	−4.99	99
3	424.59	2	5	5.53	0.66	−4.47	92
4	356.36	1	8	2.94	-0.49	−3.70	88
5	496.32	0	9	5.13	0.18	−4.02	85
6	498.47	2	8	4.26	-0.12	−5.24	84
7	499.94	1	7	4.95	0.41	−3.49	80
8	446.51	1	5	5.80	0.05	−3.98	74
9	384.64	1	5	4.36	0.76	−3.99	70
10	356.83	1	4	4.35	0.08	−4.01	65

[a] Server FAFDrugs [44], filter Drug-like soft was used: MW 100–600; HBD $\leq$ 5; HBA $\leq$ 12; LogP −3 to 6. [b] Server Molsolf [45], Drug-Likeness score was determined, values between −1 to 2 are accepted.

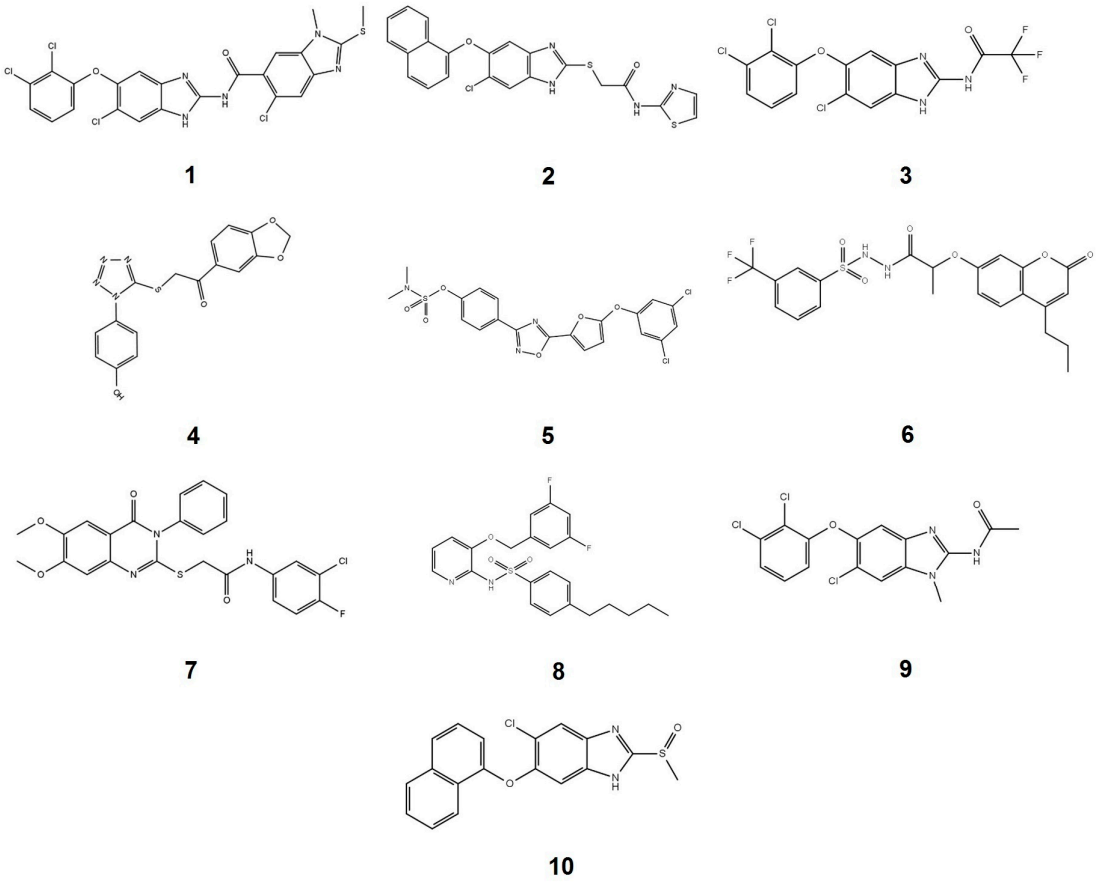

Figure 1. Chemical structures of the ten most potent PTP1B inhibitors. The number of each compound corresponds to that indicated in Table 1.

Regarding molecules **5**, **6** and **8** they have in their structure substituents that have been reported before in PTP1B inhibitors [28]. Molecules **4** and **7** represent a new chemical nucleus, which extends the structural diversity of molecules that inhibit PTP1B reported to date. Finally, molecules **1**, **2** and **3** were selected to characterize their inhibition mechanism by enzymatic kinetics, docking and molecular dynamics.

2.2. Kinetic Studies

Analysis of the plots at different substrate and fixed inhibitor concentrations indicated that the three compounds showed a mixed type inhibition mechanism (Figure 2a–c). This suggests that the three molecules are able to recognize both the free enzyme and the enzyme-substrate complex, generating the enzyme-substrate-inhibitor ternary complex, which is inactive [46] (Figure 2d). Something interesting to highlight is that compound **1** had an α value close to 1 suggesting that it recognizes, almost with the same affinity, both the free enzyme and the enzyme-substrate complex [46], whilst in compounds **2** and **3** this value was close to 3, suggesting a three times lower affinity with respect to the enzyme-substrate complex. These results suggest that the noncompetitive component of these inhibitors is stronger than the competitive component into inhibition mechanism, where the values of Ki and IC_{50} in our study were very similar [47]. The kinetic parameters were obtained from the adjustment of the data to the correspondent equation (Table 2).

The ChEMBL database from the Bioinformatics European Institute [48], reported 5854 compounds active against PTP1B. Of interest of this work, 30 of them show a reported mixed type inhibition mode, but only eight presented a Ki value lower than 5 μM [49–61]. Something important is that the chemical structures of these inhibitors are totally different from those reported here, and include DMFS derivatives [58], benzoic acid-based derivatives [57], insulin-mimetic selaginellins [59], pentacyclic acid triterpenoids [52], and oleanilic acid derivatives [61]. Also interesting is that α value reported was in the same range as for compounds **1**, **2**, and **3**.

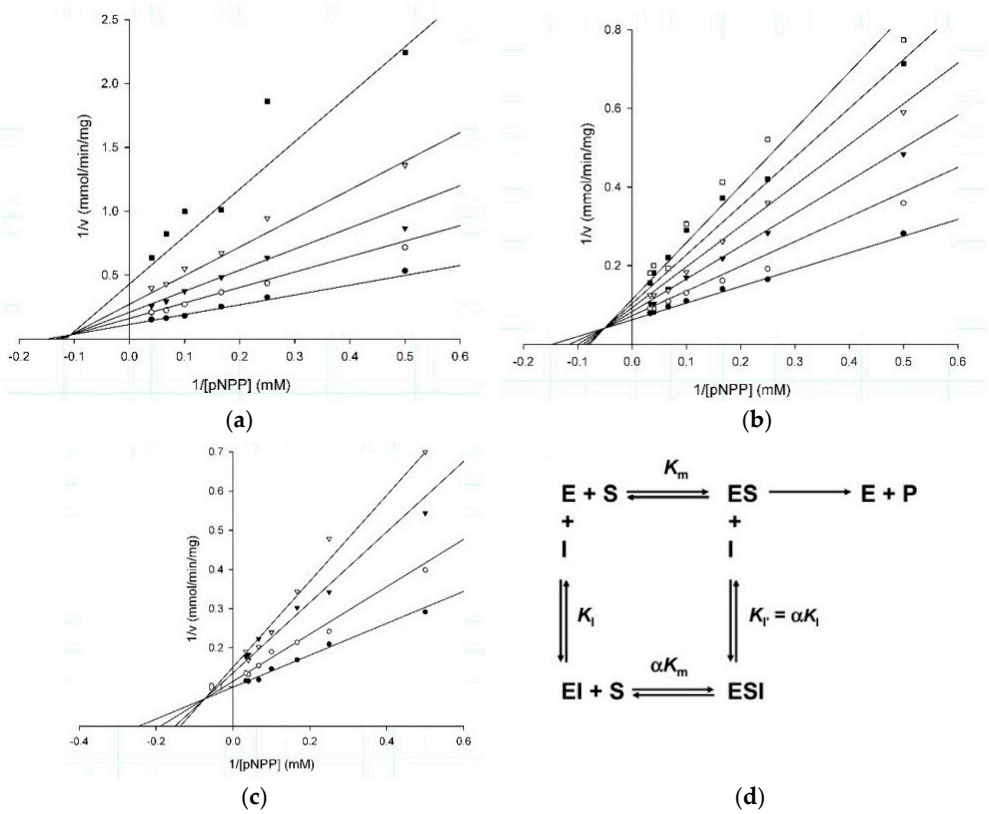

Figure 2. Lineweaver-Burk plots of (**a**) compound **1** at 0 (filled circles), 3 (open circle), 6 (filled triangle), 10 (open triangle), and 20 μM (filled squares); (**b**) compound **2** at 0 (filled circles), 2 (open circle), 4 (filled triangles), 6 (open triangles), 8 (filled squares), and 10 μM (open squares); and (**c**) compound **3** at 0 (filled circles), 20 (open circle), 50 (filled triangles), and 70 μM (open triangles); (**d**) kinetic model for mixed type inhibition. In the scheme, E corresponds to free enzyme; S is the substrate; ES is the enzyme-substrate complex; EI corresponds to the enzyme-inhibitor complex; ESI is the enzyme-substrate-inhibitor ternary complex; and P is the reaction product.

Table 2. Type of inhibition and kinetic parameters for PTP1B inhibitors.

Molecule	Ki (μM)	IC_{50} (μM)	α	V_{max} (μmol/min/mg)	Km (mM)	Inhibition Type
1	5.2	7.5	1.4	8.8	6.7	Mixed
2	4.2	8.4	2.9	16	6.8	Mixed
3	41.3	31.3	3.3	10	4.1	Mixed

With respect to available drugs for the treatment of type 2 diabetes, classified in a general way as sulfonylureas, meglitidines, biguanides, thiazolidinediones, α-glucosidase inhibitors, glucagon-like peptide-1 receptor agonists, dipeptidyl peptidase-4 inhibitors, sodium glucose transporter-2 inhibitors, synthetic amylin analogues, and dopamine-2 agonists [62,63], none of them are benzimidazole derivatives, on the contrary, they are made up of different chemical groups such as sulfonylureas, guanidines, thiazolidinediones, disaccharides, glucose derivatives, peptidomimetics, among others [63]. The above makes the compounds **1**, **2** and **3** novel structural proposals.

After kinetic studies, the binding mode and the type of interactions between the inhibitors and the PTP1B were analyzed by docking and molecular dynamics studies.

2.3. Molecular Docking

Before the molecular docking of the inhibitors, the protocol was validated through the binding mode of the inhibitor reported in the crystallographic structure used [64]. The RMSD value obtained from the modeling using Glide and the crystallographic complex was of 0.24 Å, which indicated that the docking protocol was done correctly (data not shown). After this, the three inhibitors were docked, obtaining binding energies of −4.2, −5.0 and −4.5 Kcal/mol for compounds **1**, **2**, and **3**, respectively.

As for their binding modes, the three compounds formed interactions with residues from the secondary binding site to phosphate (Arg24, Arg254, Gly259, Gln262 and Asp48) [65]. In the three cases, the molecules block the cavity of the catalytic site without interacting with the signature residues of phosphatases.

The interaction with Asp48 has been reported before in different crystallographic structures of PTP1B in complex with other benzimidazole derivatives [41–43]. Additionally the interaction of the benzimidazole nucleus with the Gln262 found in the three inhibitors has been also reported [66,67], (Figure 3). A more detailed analysis of the interactions between these molecules and the enzyme were performed by molecular dynamic studies, the results are described below.

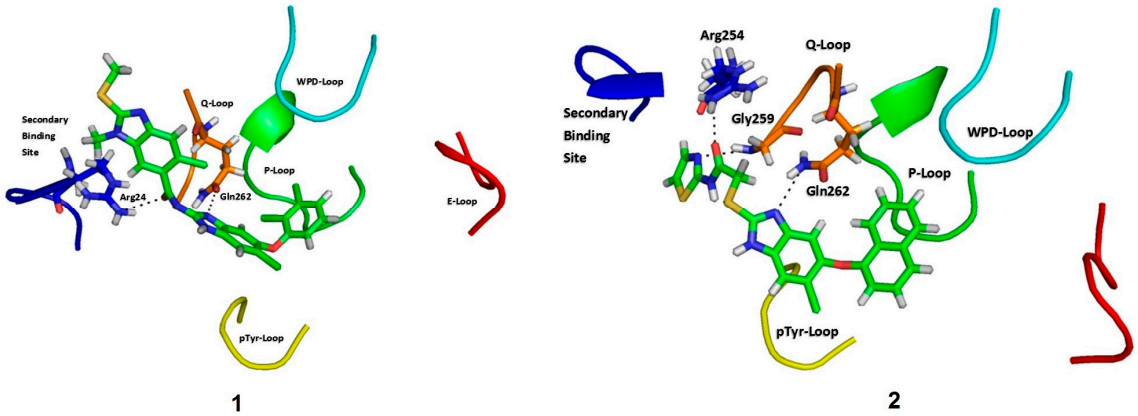

Figure 3. *Cont.*

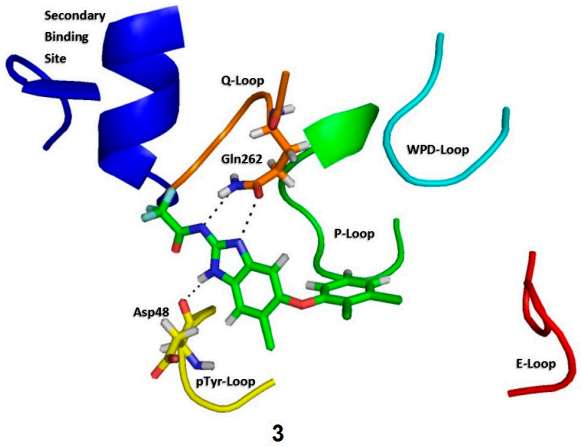

Figure 3. Binding mode of compounds **1**, **2** and **3** in PTP1B. Loops are highlighted as follows: P loop (green), WPD loop (cyan), Q262 loop (orange), pTyr46 loop (yellow), and E loop (red). Secondary phosphate binding site is highlighted in blue.

2.4. Molecular Dynamics Simulations

The complexes obtained by molecular docking were submitted to a simulation of 10 ns. The total energy variation plots showed that the energy variation was around −12 Kcal/mol, which indicate that the average energy remains constant and there is structural stability of the complexes (Figure S1). The RMSD analysis showed that the three complexes had fluctuations in the first 3500 ps, achieving the stability from the 4000 ps up to the 10,000 ps without exceeding a 0.30 Å variation (Figure S2). It also was observed the influence of the inhibitors over the protein, with the variation of the RMSD in comparison with the protein alone.

The analysis of the binding energies showed that the compound **1** presented the best global binding energy, followed by compounds **2** and **3**, which is in accordance with the inhibitory activity observed in the kinetic studies. The same situation was repeated in the individual values of the different components of the global energy, except in the electrostatic one, where compound **2** obtained the highest value (Table 3).

Table 3. Binding free energies determined by the MMPBSA method, and hydrogen bonds of the protein-ligand complexes.

Complex	Energy (kcal/mol)					Hydrogen Bonds	
	Van der Waals Energy	Electrostatic Energy	Polar Solvation Energy	SASA Energy	ΔG Binding	Range	Average
PTP1B-**1**	−47.56	−17.97	32.34	−4.17	−37.36	0–3	3
PTP1B-**2**	−35.46	−27.01	33.57	−3.54	−32.43	0–5	4
PTP1B-**3**	−29.50	−3.63	12.33	−2.93	−23.74	0–4	2

The structural analysis along the simulation time showed that the three molecules formed interactions, being the most important, the hydrophobic type interaction with the Asp48 and compound **1** (70% of occupancy), meanwhile compound **2** interacted with Phe182 (87% of occupancy). Compound **3** formed hydrogen bonds with Ala264, Gln262, and Arg24, as well as a hydrophobic interaction with Phe182, all of them with occupancy of 40% (Figure 4). Something interesting to highlight is that the interactions formed by the three inhibitors include important residues for the enzyme function like Gln262 and the Asp48, without having interactions with the denominated signature residues of the phosphatases (H/V)CXXGXXR(S/T) [34,68]. In this context, several studies have shown that selectivity against TCPTP can be achieved by interactions with residues such as Arg24, Arg47, Asp48, Arg254, Met258, and Gln262 [35,65]. Additionally, we investigated their binding mode

in TCTPT by molecular docking, finding that the three compounds made interactions with different residues of the enzyme (Figure S3). In conclusion, computational studies suggest that these inhibitors could be selective for PTP1B.

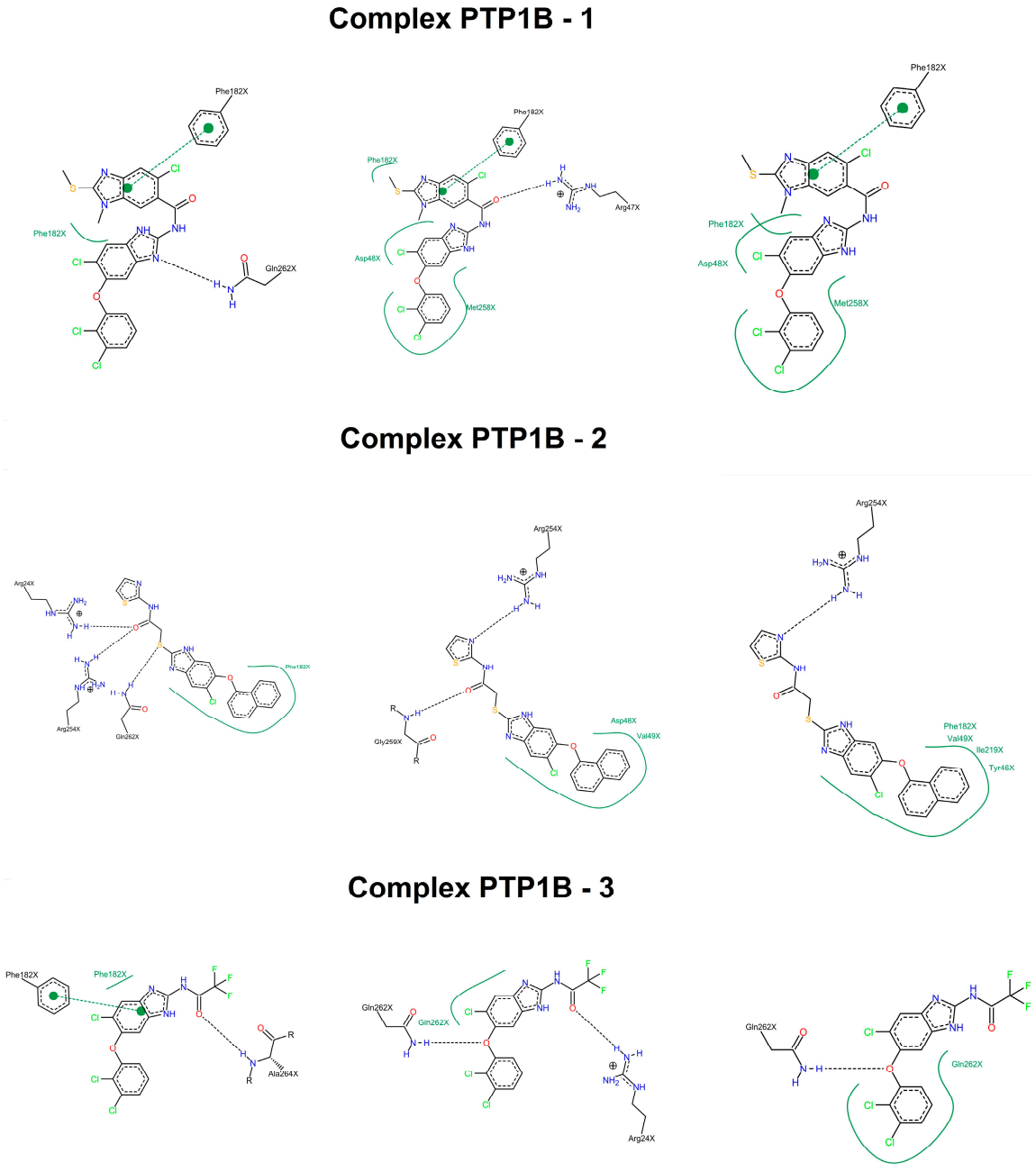

Figure 4. Two dimensional maps of interaction from the complexes PTP1B-**1**, PTP1B-**2,** and PTP1B-**3**. The image shows interactions at different times during entire dynamics: beginning, stabilized (4 ns), and final (10 ns). Hydrogen bonds between protein and ligand are drawn as dashed lines. Hydrophobic contacts are represented by means of spline sections highlighting the hydrophobic parts of the ligand and the name of the contacting amino acid. Maps were generated in Server Poseview (http://proteinsplus.zbh.uni-hamburg.de/#poseview).

2.5. Physicochemical and Drug-Like properties

The in silico evaluation of the physicochemical and drug-like parameters suggested that these molecules possess the necessary chemical features to potentially have an acceptable oral absorption [69–71] (Table 1 and Figure 5).

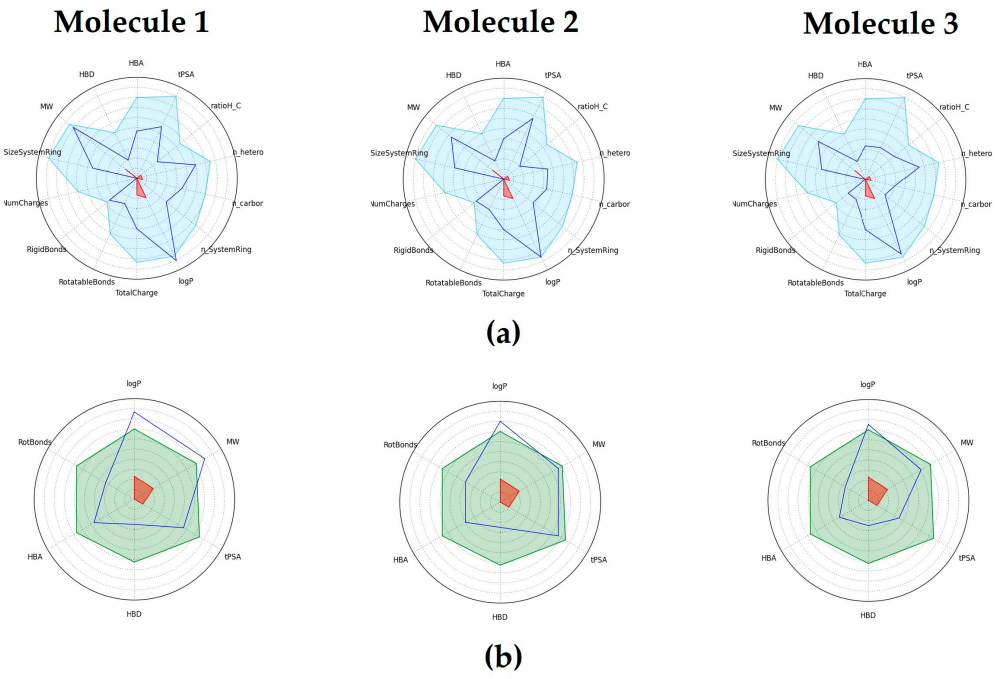

Figure 5. (**a**) Scheme of PhysChem Filter Positioning, compound values (blue line) should fall within the drug-like filter area (light blue); (**b**) Scheme of Oral Absorption Estimation, compound values (blue line) should fall within RO5 and Veber rules area (light green). The logarithm of the partition coefficient between n-octanol and water, logP; Molecular Weight, MW; Hydrogen Bond Donnors, HBD; Hydrogen Bond Acceptors, HBA; topological Polar Surface Area, tPSA; het/carbon atoms ratio, ratioH/C; Number of Heteroatoms, n_hetero; Number of Carbon Atoms, n_carbor; Number of Smallest Set of Smallest Rings, n_SystemRing.

2.6. Toxicological Evaluation

An important point to analyze during the development of any new drug is the toxicological profile of the molecule. In this matter, using different softwares available online, a detailed study to predict the toxicological potential of these three inhibitors was performed. The estimation of the lethal dose 50 (LD_{50}) suggested that the three molecules are moderately toxic. Nevertheless, according to software evaluation, there are no toxic fragments reported in their structure, neither biological targets that can denote toxicity. With respect to their mutagenic, tumorigenic, irritability and reproductive effects, the only molecule that did not show any of these features was compound **2** (Table 4).

Taking into account all the data presented, compound **2** is the most viable option to continue with its optimization since it showed the best kinetic and predicted physicochemical and toxicological features. However, compounds **1** and **3** are still interesting structures that provide important information for the design of new inhibitors.

Table 4. Toxicological profile of PTP1B inhibitors.

Molecule	LD50 [a] (mg/kg)	Toxicity Class [a]	Toxic Frag. [a]	Toxicity Targets [a]	Mutagenic [b]	Tumorigenic [b]	Reprod. Effec. [b]	Irritant [b]	Drug Likeness [c]
1	1600	4	None	No Binding	Low	None	High	High	1.07
2	1000	4	None	No Binding	None	None	None	None	0.86
3	1600	4	None	No Binding	Low	None	High	High	0.66

[a] Toxicity Class was determined in Server PROTOX [72], values ranged between 1 to 6, 1 is toxic and 6 is safe. Toxicity targets were determined for: Adenosine A2A receptor, Adrenergic beta 2 receptor, Androgen receptor, Amine oxidase, Dopamine D3 receptor, Estrogen receptor 1 and 2, Glucocorticoid receptor, Histamine H1 receptor, Nuclear receptor subfamily 1 group I member 2, Opioid receptor kappa, Opioid receptor mu, cAMP-specific 3′,5′-cyclic phosphodiesterase 4D, Prostaglandin G/H synthase 1, Progesterone receptor. [b] Mutagenic, Tumorigenic, Reproductive effective and Irritant effects were determined using Data Warrior [73]. [c] Drug-Likeness score was determined with Server Molsolf [45], values between −1 to 2 are accepted.

3. Materials and Methods

3.1. General Information

The reagents used were purchased from Sigma-Aldrich (St. Luis, CA USA), kinetic analysis were performed in a diode array spectrophotometer model 8453 from Agilent (Santa Clara, CA, USA).

3.2. Compounds

The tested chemical library was composed of an in-house set of 100 compounds and 150 small molecules of the Fragment Library and HitFinder™ collection from Maybridge (Waltham, MA, USA). Compounds **4** to **8** belong to Maybridge with the identification codes HTS 01664 for ′1-(1,3-benzodioxol-5-yl)-2-{[1-(4-hydroxyphenyl)-1*H*-1,2,3,4-tetraazol-5-yl]sulfanyl}-1-ethanone (**4**); SP 00892 for 4-{5-[5-(3,5-dichlorophenoxy)-2-furyl]-1,2,4-oxadiazol-3-yl}phenyl-*N*,*N*-dimethylsulfamate (**5**); RJF 01991 for ′*N*′1-{2-[(2-oxo-4-propyl-2*H*-chromen-7-yl)oxy]propanoyl}-3-(trifluoromethyl)benzene-1-sulfonohydrazide (**6**); HTS 02534 for ′*N*-(3-chloro-4-fluorophenyl)-2-[(6,7-dimethoxy-4-oxo-3-phenyl-3,4-dihydro-2-quinazolinyl)sulfanyl]acetamide (**7**); RH 02067 for ′*N*-{3-[(3,5-difluorobenzyl)oxy]pyridin-2-yl}-4-pentylbenzenesulfonamide (**8**). The general synthesis method for compounds **1**, **2**, **3**, **9** and **10** is outlined below.

Compounds **1**–**3** and **9** were prepared from the appropriate benzimidazole-2-amine and the adequate acid or anhydride under the guidelines of our synthetic procedure previously reported for similar benzimidazole derivatives [74,75]. Briefly, for compound **1**: the substituted benzimidazol-2-amine was reacted with 5-chloro-1-methyl-2-(methylthio)-6-carboxylic acid, previously treated with 1,1′-carbonyldiimidazole in DMF at room temperature for 2 h; then, the reaction mixture was heated at 140 °C under MW irradiation for 30 min. For compounds **3** and **9**: the substituted benzimidazol-2-amine was reacted with trifluoroacetic anhydride (compound **3**) or acetic anhydride (compound **9**) at 0 °C to r.t. in CH_2Cl_2 or $CHCl_3$; compound **2** was prepared from 6-chloro-5-(1-naphtyloxy)-1*H*-benzimidazole-2-thiol [76] and 2-chloro-*N*-(thiazol-2-yl)acetamide in acetone at 0 °C [77]. Compound **10** was obtained as previously reported [78].

5-Chloro-N-[6-chloro-5-(2,3-dichlorophenoxy)-1H-benzimidazol-2-yl]-1-methyl-2-(methylthio)-1H-benz-imidazole-6-carboxamide (**1**). Recrystallized from DMF/MeOH white solid (89%); m.p. 269-270 °C. ^{1}H-NMR (DMSO-d_6; 400 MHz): δ 2.75 (s, 3H, S-CH_3); 3.72 (s, 3H, N-CH_3); 6.69 (dd, 1H, *J* = 8.2 Hz, 1.2 Hz, H-6 dichlorophenoxy); 7.27 (t, 1H, *J* = 8.2 Hz, H-5 dichlorophenoxy); 7.33 (s, 1H, H-4′); 7.37 (dd, 1H, *J* = 8.4 Hz, 1.2 Hz, H-4 dichlorophenoxy); 7.68 (s, 1H, H-7′); 7.71 (s, 1H, H-5); 7.91 (s, 1H, H-7); 12.40 (bs, 1H, NH, int. D_2O). ^{13}C-NMR (DMSO-d_6; 100 MHz): δ 14.48 (S-$\underline{C}H_3$); 30.56 (N-$\underline{C}H_3$); 110.93 (C-7 benzimidazole); 115.64 (C-6 dichlorophenoxy); 118.45 (C-4 benzimidazole); 118.59 (C-7′a benz-imidazole); 121.36 (C-2 dichlorophenoxy); 123.73 (C-5 benzimidazole); 124.69 (C-4 dichlorophenoxy); 127.79 C-6 benzimidazole); 129.11 (C-5 dichlorophenoxy); 133.23 (C-3 dichlorophenoxy); 135.63 (C-7a benzimidazole); 144.89 (C-3a benzimidazole); 145.04 (C-6′ benzimidazole); 148.58 (C-2′ benzimidazole); 155.10 (C-1 dichlorophenoxy); 157.27 (C-2

benzimidazole); 166.85 (C=O amide). EI-MS: *m*/*z* 565 (M^+); HRMS (FAB^+): 565.9752 $[M + H]^+$ (Calcd for $C_{23}H_{15}O_2N_5Cl_4SH^+$ 565.9773).

2-[6-Chloro-5-(1-naphthalyloxy)-1H-benzimidazol-2-yl]thio-N-(thiazol-2-yl)acetamide (**2**). Recrystallized from methanol to give a beige solid (20% yield); m.p. 155-157 °C. ^{1}H-NMR (400 MHz, DMSO): δ 4.37 (s, 2H, -CH_2-). 6.64 (d, *J* = 7.6 Hz, 1H, H-2 naphtyloxy), 7.23 (d, *J* = 3.6 Hz, 1H, H-5 thiazolyl), 7.27 (s, 1H, H-7), 7.37 (t, *J* = 8.0 Hz, 1H, H-3 naphtyloxy), 7.48 (d, *J* = 3.6 Hz, 1H, H-4 thiazolyl), 7.61–7.57 (m, 2H, H-6 y H-7 naphtyloxy), 7.64 (d, *J* = 8.0 Hz, 1H, H-4 naphtyloxy), 7.72 (s, 1H, H-4), 7.99–7.95 (m, 1H, H-5 naphtyloxy), 8.27–8.22 (m, 1H, H-8 naphtyloxy), δ 12.70 (s, 1H, CONH). ^{13}C-NMR (DMSO-d_6; 100 MHz): δ 34.79 (SCH_2), 109.88 (C-2 naphtyloxy), 113.78 (C-3 naphtyloxy), 119.12 (C-5 thiazolyl), 121.36 (C-8 naphtyloxy), 122.45 (C-4 naphtyloxy), 124.86 (C-5 or C-6), 126.02 (C-6 or C-7 naphtyloxy), 126.13 (C-6 or C-7 naphtyloxy), 126.85 (C-4a or C-8a naphtyloxy), 127.77 (C-5 naphtyloxy), 134.45 (C-4a or C-8a naphtyloxy), 137.76 (C-4 thiazolyl), 146.06 (C-3a or C-7a), 151.96 (C-3a or C-7a), 153.37 (C-2), 157.81 (C-2 thiazolyl), 166.22 (CONH). MS (DART): *m*/*z* (%): 467 ($[M + H]^+$, 15). HRMS (DART): Calcd for $C_{22}H_{15}ClN_4O_2S_2$ $[M + H]^+$: 467.04032, found: 467.04177.

N-[6-Chloro-5-(2,3-dichlorophenoxy)-1H-benzimidazol-2-yl]-2,2,2-trifluoroacetamide (**3**). Purified by washing with cold water. Beige solid; m.p. > 200 °C (d). ^{1}H-NMR (400 MHz, DMSO) δ: 6.84 (dd, 1H, J_1 = 8.3, J_2 = 1.3 Hz, H-6′); 7.21 (s, 1H, H-4); 7.32 (t, 1H, *J* = 8.2 Hz, H-5′); 7.44 (dd, 1H, J_1 = 8.1 Hz, J_2 = 1.3 Hz, H-4′); 7.66 (s, 1H, H-7); 13.07 (bs, 1H, CO-N*H*). ^{13}C-NMR (DMSO-d_6; 100 MHz): δ 105.22 (C-4), 114.10 (C-7), 116.97 (C-6′), 117.52 (q, J_{F-C} = 287 Hz, -CF_3), 120.37 (C-6), 122.15 (C-2′), 125.65 (C-4′), 127.03, 129.22, 129.36 (C-5′), 133.41 (C-3′), 147.07 (C-5), 153.98 (C-2), 154.17 (C-1′), 162.99 (q, J_{F-C} = 35 Hz, CO).

N-[6-Chloro-5-(2,3-dichlorophenoxy)-1-methyl-1H-benzimidazol-2-yl]acetamide (**9**). Recrystallized from ethanol, white crystals (84% yield); m.p. 237.5–238.9 °C. ^{1}H-NMR (400 MHz, DMSO) δ: 2.16 (s, 3H, CO-CH_3); 3.64 (s, 1H, N-CH_3); 6.65 (d, 1H, J_1 = 8.2 Hz, J_2 = 0.9 Hz, H-6′); 7.24 (t, 1H, *J* = 8.2 Hz, H-5′); 7.36 (dd, 1H, J_1 = 8.1 Hz, J_2 = 1.3 Hz, H-4′); 7.42 (s, 1H, H-4); 7.88 (s, 1H, H-7); 10.97 (bs, 1H, CO-NH). ^{13}C-NMR (DMSO-d_6; 100 MHz): δ 23.43 (CO-CH_3), 30.97 (N-CH_3), 111.95 (C-4), 112.27 (C-7), 115.51 (C-6′), 119.52 (C-6), 121.28 (C-2′), 124.70 (C-4′), 129.16 (C-5′), 133.25, 140.41 (C-3′), 145.39 (C-4), 148.37 (C-2), 155.14 (C-1′), 170.57 (CO). EA. Calc.: $C_{16}H_{12}Cl_3N_3O_2$: C, 49.96; H, 3.14; N, 10.9. Found: C, 49.82; H, 2.57; N, 10.6. HMRS (ESI) Calcd for $C_{16}H_{12}Cl_3N_3O_2$ [M + Na]: 406.0073; found 406.

3.3. Expression and Purification of PTP1B

The region of the gene PTPN1 that encodes for PTP1B (residues 1–321) was synthetized by Integrated DNA Technologies and inserted in the pIDTSmart plasmid. Then, the gene was liberated by restriction reactions using Ndel and BamH1 enzymes and inserted into the overexpression vector pET28A. Afterwards, *E. coli* BLR strains were transformed for the overexpression of the protein. With this purpose, 500 mL of LB liquid culture medium was grown supplemented with Kanamycin (50 μg/mL) at 37 °C, once it reached an optical density of 0.9 at 600 nm, 1mM of IPTG was added to induce the overexpression, incubating four more hours. Right away, cells were cultured by centrifugation and lysed by sonication. The supernatant was passed through a Ni-agarose column and the enzyme was purified by an imidazole gradient. The fractions were analyzed by SDS-PAGE electrophoresis and those with the presence of the protein were pooled and concentrated with a Plus-70 centricon, immediately the enzyme was precipitated with ammonium sulfate (80% saturation).

3.4. Enzymatic Activity

The PTP1B activity was measured based on the Goldstein method [79]. The assay was performed with a final reaction volume of 500 μL in HEPES buffer (50 mM HEPES, 1mM DTT, 2 mM EDTA and 150 mM NaCl, pH 7.0), DMSO (10%) and p-nitrophenol phosphate (pNPP) as substrate (50 mM), the reaction was started with the PTP1B (1.5 μg/mL). After 30 min of incubation at 37 °C, the reaction

was stopped by the addition of 500 µL of NaOH 5N reading the absorbance at 405 nm. The number of hydrolyzed moles of pNPP was determined using the molar extinction coefficient of the product pNP (18,500 M^{-1} cm^{-1}).

3.5. Inhibition Assays

Inhibition assays were performed under the above described conditions, adding to the reaction each one of the molecules at a final concentration of 200 µM. The concentration that inhibits 50% (IC_{50}) of the PTP1B activity was determined through curves at different concentration of each compound, adjusting the data to the equation reported elsewhere [80]. The inhibition type and constant were obtained by the measurement of the initial velocities of hydrolysis varying the substrate concentration in a range of 2–30 µM, in absence or presence of fixed concentrations of each inhibitor. For compound **1** the concentrations used were 3, 6, 10 and 20 µM; in the case of compound **2** were 2, 4, 6, 8, and 10 µM; and for compound **3** were 20, 50, 70 and 90 µM. The experimental data were analyzed through the Lineweaver-Burk plot using the software Sigma Plot V12.3 (Systat Software, Inc., San Jose, CA, USA).

3.6. Molecular Docking

The molecules were built in Maestro 10.4 (www.schrodinger.com) and prepared in Ligprep 2.3 [81]. The crystallographic structure of the PTP1B protein was obtained from the RCSB Protein Data Bank with the code PDB ID 2F71 [64]. Hydrogen atoms were added to the structure, bond angles, and distances were corrected, and charges were assigned using Protein Preparation Wizard [82], all ions and the inhibitor present in the crystallographic structure were withdrawn. Water molecules were also withdrawn, with exception of those located in the WPD loop, since these are considered necessary to give better binding poses [83]. Energy minimization was performed with the OPLS_2005 force field with an RMSD of 0.3 Å. The molecular docking simulations in the active site of the PTP1B were performed using Glide [84,85]. The Van der Waals scale was of a factor of 0.80 and a cutting partial charge of 0.15. The files were limited to at least one pose for ligand, rejecting poses with energies smaller than 0.5 kcal/mol. Standard precision and Extra precision modes were used with flexible ligand adding penalization states of the Epik software [86] in the docking score.

3.7. Molecular Dynamic Simulations

The initial structures for the simulations were those with the lowest binding energy of each complex obtained by docking. The necessary topology files for each ligand (compounds **1**, **2** and **3**) were calculated and obtained using PRODRG [87]. The systems were solvated within a water box with 1.0 nm of distance from the proteins surface with the Single Point Charge (SPC) water model. Sodium and Chlorine ions were added to neutralize the systems charge until a 0.15 M concentration was reached. First, a descending steps energy minimization was done. Afterwards, a canonic assemble was performed, continuing by an isobaric-isothermal assemble, maintaining a constant temperature, volume and pressure. Finally, 10 ns simulations were performed for each complex and the free enzyme in GROMACS 5.1 software [88] using the Gromos 43a147 force field. All simulations were performed at 1 bar of pressure and 300 °K of temperature. The free binding energy was calculated based on the molecular mechanics of surface area of Poisson-Boltzmann (MM-PBSA) method [89].

3.8. Drug-Like and Toxicological Propierties

The FAF-Drugs4 server [42] and the Molsoft [45] program, available on the web were used. The Drug-like soft filter used in FAF-Drugs4 combines the physicochemical properties described in several articles and an analysis of the descriptor values of 916 oral medications of the FDA, allowing defining a filter threshold that comprises up to 90% of these drugs. The ranges of the permitted values used by the software are shown in Table 1. Regarding Molsoft, it uses the fingerprints technique with a set of 5000 commercialized drugs and 10,000 non-pharmacological compounds. After the process, it reports a score that places the molecules in a range between the parameters of the drugs

and the non-drugs, which allows defining their pharmacological potential; values between −1 to 2 are accepted.

With respect to toxicological parameters, PROTOX [72] server was used, as well the Data Warrior software [73] to determine mutagenesis, tumorigenic, reproductive effects and irritability. PROTOX uses the identification of fragments over-represented in toxic compounds and similarity analyses of compounds with known LD50 values. Furthermore, based on pharmacophores, it indicates possible toxicity targets. Data Warrior uses chemical descriptors to make several molecular similarity measures and predict properties such as mutagenicity, tumorigenicity, irritant and reproductive effects.

4. Conclusions

In the present work three new mixed type inhibitors for PTP1B are reported, which based on their inhibition capability and mechanism, potential selectivity against TCPTP, and predicted drug-like properties, could represent a good starting point for the development of more potent molecules that can guide the design of a new drug to treat type 2 diabetes.

Supplementary Materials: Supplementary materials are available online.

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Author Contributions: Performed the experiments: M.J.S.-S., P.J.T.-S., J.M.V.-L., and C.C.-G. Conceived and designed in-house compounds library: R.C. and A.H.-C. Conceived, designed the experiments and contributed reagents/materials/analysis tools: R.C., A.H.-C., C.A.-D., E.S.-C., J.M.S.-P., and A.T.-V. Analyzed the data and wrote the paper: M.S.-S., D.E.-M., C.A.-D., E.S.-C., M.V.-S., and A.T.-V.

Conflicts of Interest: The authors declare no conflict of interest.

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RESEARCH ARTICLE Open Access

Hypocholesterolemia is an independent risk factor for depression disorder and suicide attempt in Northern Mexican population

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Abstract

Background: Cholesterol has been associated as a risk factor for cardiovascular disease. Recently, however, there is growing evidence about crucial requirement of neuron membrane cholesterol in the organization and function of the 5-HT_{1A} serotonin receptor. For this, low cholesterol level has been reported to be associated with depression and suicidality. However there have been inconsistent reports about this finding and the exact relationship between these factors remains controversial. Therefore, we investigated the link between serum cholesterol and its fractions with depression disorder and suicide attempt in 467 adult subjects in Mexican mestizo population.

Methods: Plasma levels of total cholesterol, triglycerides, and high-density lipoprotein cholesterol (HDL-c) and low density lipoprotein cholesterol (LDL-c) were determined in 261 MDD patients meeting the DSM-5 criteria for major depressive disorder (MDD), 59 of whom had undergone an episode of suicide attempt, and 206 healthy controls.

Results: A significant decrease in total cholesterol, LDL-cholesterol, VLDL-cholesterol and triglyceride serum levels was observed in the groups of MDD patients and suicide attempt compared to those without suicidal behavior ($p < 0.05$). After adjusting for covariates, lower cholesterol levels were significantly associated with MDD (*OR* 4.229 *CI* 95% 2.555 – 7.000, $p<.001$) and suicide attempt (*OR* 5.540 *CI* 95% 2.825 – 10.866, $p<.001$)

Conclusions: These results support the hypothesis that lower levels of cholesterol are associated with mood disorders like MDD and suicidal behavior. More mechanistic studies are needed to further explain this association.

Keywords: Suicide attempt, depression, cholesterol

Background

Suicide is one of the most disastrous outcomes of psychiatric disorders [1]. It is a significant public health problem and is one of the leading causes of death worldwide [2–4]. In Mexico the rate of suicide is a current health problem that is accentuated by the fact that it is a country with an emerging market economy. In recent years, Mexico has presented an increase in its suicide rate. For example, between 2000 and 2014 there was an increase in the suicide rate from 3.5 to 5.2 cases per 100,000 inhabitants (http://bibliodigitalibd.senado.gob.mx/handle/123456789/3181) [5].

Suicidal behavior includes a wide spectrum of behaviors, such as completed suicides, high-lethality suicide attempts, and low-lethality suicide attempts [6, 7]. Although roughly 60% of all suicides occur in the context of depressive disorders [7, 8] it is still challenging for clinicians to predict suicide risk in patients with depression. For this reason, increased attention has been paid to potential biomarkers for suicide in patients with major depressive disorder (MDD) and suicidal behavior [1, 9].

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Many studies have confirmed that biological markers might be linked to suicidality, among which serum lipid levels might play an important role [2, 10–12]. However, there has been considerable controversy about the association between serum lipid levels and suicidality. Some human studies showed that suicide attempters had lower cholesterol levels [2, 11, 13, 14]others reported positive associations between cholesterol and completed suicide[2, 15–17]and some others indicated that there was no evidence for an association between serum cholesterol and suicidality [18, 19].Although the mechanism behind hypocholesterolemia and suicide has not been clearly defined, previous studies have suggested that altered cholesterol at synaptic lipid rafts may cause a reduction in serotonin communication [2].

In the Mexican population, very few studies have examined the association between serum lipid levels and the presence of depression and suicide attempt, and none have reported the frequency of hypocholesterolemia in depressed patients and suicide attempters. Here, we hypothesized that: 1) serum lipid levels are reduced in subjects with depression and suicide attempt; 2) Hypocholesterolemia is a risk factor associated with depression and suicide attempt.

Methods

Study population

After the approval of protocol by Research and Ethics Committee of the General Hospital 450 (No. 12/0306-2015), a case-control study was conducted with 261 MDD adult patients, 59 of these also had a recent episode of suicide attempt and 206 healthy adult volunteer controls of both genders; all subjects provided informed consent. Study subjects were recruited from the Mental Health Hospital "Dr. Miguel Valle Bueno" (Secretary of Health) and psychiatry service of the General Hospital 450 (Secretary of Health) and General Hospital "Dr. Santiago Ramón y Cajal" (ISSSTE), in Durango City, Mexico, from June2015to December 2016.

MDD diagnosis was made by trained psychiatrists according to DSM-5 criteria. We defined suicide attempt as "a non-fatal, self-directed, potentially injurious behavior with an intent to die as a result of the behavior" as defined by the Center for Disease Control and Prevention (https://www.cdc.gov/violenceprevention/suicide/definitions.html) [20]. For our recruitment, we considered only participants that required hospitalization.Subjects in the case group were matched with subjects in the control group based on age, sex and body mass index (BMI).The latter, in order to exclude nutritional state as a confounding factor and due to previous association between BMI and cholesterol levels. [21]. Control subjects were apparently healthy persons without any symptoms or signs of MDD based on a clinical examination at inclusion. Use of lipid lowering drugs, such as statins was considered a confounding factor due to the diverse effects it may have on cellular mediation of inflammation and immunity [22] in conjunction with its known effect on depression [23] and was, therefore, an exclusion criterion. Lastly, we excluded those with chronic diseases (hepatic disorders, diabetes mellitus, hypertension, cardiovascular disease) due to their association with dyslipidemia. [24, 25]

Lipid profile

Blood samples were obtained by venous puncture of all participants after an overnight fasting period. The lipid profiles were determined using the A15 Clinical Chemistry and Turbidimetry Systems (BioSystems) according to the manufacturer's instructions. The level of serum lipids was evaluated based on total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG).Hypocholesterolemia was defined as a total cholesterol concentration of less than 150 mg/dl.

Statistical Analyses

Numerical data were presented as mean±standard deviation (*SD*). Categorical variables were presented as proportions. Differences between the two case groups (MDD and MDD associated with suicide attempt) and control group (healthy subjects) were estimated using ANOVA (Kruskall Wallis test for skewed data) for numerical variables with an additional Bonferroni post-hoc test, and the chi-squared test for categorical variables. Additionally, our analysis was stratified by sex and logistic regression analysis was performed to determine the association between hypocholesterolemia (independent variable) and the presence of depression and suicide attempt (dependent variables). Values of $p<0.05$ were considered statistically significant. The Statistical Package for the Social Sciences (SPSS) for Windows version 21.0 (SPSS Inc., Chicago, IL) was used for data management and statistical analysis. Odds ratio (*OR*) and 95% confidence interval (95%*CI*) were determined, while p value <0.05 defined the statistical significance.

Results

Clinical characteristics and laboratory parameters of the 467 enrolled subjects are shown in Table 1. The comparative analysis of the serum lipid levels between the study groups showed significant differences in the total cholesterol, LDL-cholesterol, VLDL-cholesterol, triglycerides serum levels and, hypocholesterolemia frequency ($p<0.05$). No significant difference in HDL levels were observed between groups.

Post-hoc analysis between the two groups comprised of patients with MDD with suicide attempt ($p<0.001$) and MDD only ($p<0.001$) showed significantly lower total cholesterol levels compared to the control group. Also, patients with MDD who were associated with suicide attempt had

Table 1 Mean cholesterol levels and hypocholesterolemia frequency according to mental impairment categories

	Healthy subjects	MDD	MDD associated w/suicide attempt	*P* value
n	206	202	59	–
Age, years	36.8 ± 6.6	37.3 ± 10.0	35.2 ± 10.5	0.363
Females / Males, n (%)	166 (80.5) /40 (19.5)	169 (83.7) /35 (16.3)	42 (71.2) /17 (28.8)	0.139
Obesity frequency (IMC>30 kg/m^2), n (%)	55 (26.8)	63 (32)	15 (25.4)	0.431
Total cholesterol serum level, mg/dl	172.5 ± 25.3	167.9± 45.1	152.2 ± 39.0	<.001‡,*,†
HDL cholesterol serum level, mg/dl	46.0 ± 11.6	43.8 ± 14.5	46.0± 18.7	0.198
LDL cholesterol serum level, mg/dl	96.7 ± 29.4	84.0 ± 39.5	76.9 ± 32.5	0.014*
VLDL cholesterol serum level, mg/dl	28.9± 14.8	41.2± 23.0	37.6± 21.3	<.001‡,†
Triglyceridesserum level, mg/dl	142.7 ± 87.1	208.3± 119.7	172.8 ± 88.1	<.001‡,*,†
Hypocholesterolemia frequency (<150 mg/dl), n (%)	28 (13.6)	79 (38.2)	29 (49.2)	<.001

MDD Major depression disorder
Values are mean ± standard deviation
*Statistically significant difference between Healthy subjects and MDD associated with suicide attempt
†Statistically significant difference between MDD and MDD associated with suicide attempt
‡Statistically significant difference between Healthy subjects and MDD

significantly lower total cholesterol levels compared with MDD only (p 0.016).

LDL levels were lower in the MDD associated with suicide attempt group with respect to the control group (p 0.013). Subjects with depression presented higher VLDL (p<0.001) and triglyceride levels (p<0.001) compared with healthy controls. Similarly, subjects with depression presented higher VLDL (p 0.043) and triglyceride levels (p 0.039) compared with MDD with suicide attempt. The frequency of hypocholesterolemia was significantly higher in the MDD and MDD with suicide attempt groups compared with the control group (p<0.001).

A subgroup analysis by gender was performed in order to separately assess the magnitude of the association between lipid serum levels in men and women with MDD and suicide attempt (Table 2). Both, men and women showed significantly lower total cholesterol levels in subjects with MDD associated with suicide attempt compared with the control group (p<0.05). However, only in women, did we observe a significant difference between the MDD-only

Table 2 Analysis by gender of clinical characteristics and laboratory parameters

	Male			*p* value	Female			*p* value
	Healthy subjects	MDD	MDD associated w/suicide attempt		Healthy subjects	MDD	MDD associated w/suicide attempt	
n	40	36	17		166	171	42	
Age, years	38.1 ± 6.1	35.8 ± 10.5	37.3 ± 11.9	*0.592*	36.5 ± 6.7	37.5 ± 10.0	34.3 ± 9.9	*0.133*
Obesity frequency (IMC>30 kg/m^2), n (%)	10 (25)	6 (17.1)	3 (17.6)	*0.664*	45 (27.3)	57 (34.8)	12 (28.6)	*0.322*
Total cholesterol serum level, mg/dl	182.5 ± 42.5	164.8 ± 48.6	140.2 ± 32.1	*0.002*‡,*	170.1 ± 18.4	168.5 ± 44.4	157.1 ± 40.8	*0.017**
HDL cholesterol serum level, mg/dl	46.4 ± 14.5	41.7 ± 15.8	44.1 ± 12.4	*0.566*	45.8 ± 10.2	44.2 ± 14.2	46.8 ± 20.9	*0.301*
LDL cholesterol serum level, mg/dl	100.8 ± 35.9	71.2 ± 37.2	71.7 ± 24.5	*0.011*‡	94.8 ± 26.2	86.7 ± 39.5	78.9 ± 35.3	*0.165*
VLDL cholesterol serum level, mg/dl	30.9 ± 17.0	55.4 ± 33.4	29.8 ± 10.1	*0.002*‡,†	28.1 ± 13.9	38.1 ± 18.9	34.9 ± 18.7	*0.004*‡
Triglycerides serum level, mg/dl	137.7 ± 66.9	277.1 ± 167.2	157.6 ± 60.2	<.001‡,†	143.9 ± 91.4	193.7 ± 101.8	179.0 ± 97.2	<.001‡,*
Hypocholesterolemia frequency (<150 mg/dl), n (%)	8 (20)	17 (47.2)	9 (52.9)	*0.015*	20 (12)	62 (36.3)	20 (47.6)	<.001

Values are mean ± standard deviation
*Statistically significant difference between Healthy subjects and MDD associated with suicide attempt
†Statistically significant difference between MDD and MDD associated with suicide attempt
‡Statistically significant difference between Healthy subjects and MDD

group versus the control group. No such relationship was seen in males. The comparative analysis of the hypocholesterolemia frequency between the study groups showed significant differences in both genders ($p<0.05$).

A bivariate logistic regression using data matched on age, sex, and BMI showed a statistically significant association between hypocholesterolemia and MDD (*OR* 4.229 *CI* 95% 2.555 – 7.000, $p< 0.001$). In the same way, a statistically significant association between hypocholesterolemia and suicide attempt was observed (*OR* 5.540 *CI* 95% 2.825 – 10.866, $p<0.001$). Additionally, triglycerides were analyzed and we found the following Odds Ratios and 95% Confidence Intervals: for hypertriglyceridemia in MDD [3.528 (2.326-5.352); $p<0.001$]; for hypertriglyceridemia in MDD with suicide attempt [2.626 (1.411-4.885); $p=0.002$]. Upon further analysis, no significant association in the other variables.

Discussion

Here, we show that lower serum cholesterol levels are linked with MDD and suicide attempt. Age and sex-adjusted analyses showed a clear association between serum cholesterol levels and the risk of depression and suicide attempt.

Previously, some studies have also shown an association between low cholesterol and increased risk of death due to injuries or suicide [2, 9, 26–29], but not in other reports [1, 30–33], even elevated cholesterol levels have been associated with suicide mortality in other studies [15, 33].

In Mexican subjects, there is only one research paper that has examined the possible link with hypocholesterolemia and suicide attempt in subjects with depression [34]. This study found no difference in lipid profiles between patients who had attempted suicide and those who had not. However, these authors studied only 33 patients with a major depressive episode (moderate to severe) comparing 18 subjects who had attempted suicide versus subjects who had never attempted suicide.

Although there is evidence of a link between low serum cholesterol levels and suicide in patients with depression [35, 36], the mechanism that may link serum lipids with suicidality is still unclear. It has been established that nearly all brain cholesterol is produced *in situ* through de novo synthesis and that adequate prevention of its uptake from the bloodstream is provided by selectivity of the blood-brain barrier [37–39]. Nonetheless, it is viable that decreased peripheral cholesterol in those individuals with psychiatric disorders occurs concurrently with cholesterol modifications that take place in distinct synaptic lipid rafts in neurons (by a common regulatory mechanism). This could produce the minimized activity of serotonergic communication and, consequently, give rise to instinctive responses and violent suicidal behavior [2, 40].

Cholesterol is the paramount constituent of cellular membranes in higher eukaryotes and is essential in membrane function and organization as well as dynamics and sorting. It is commonly found dispersed in a non-random form in specific areas (domains) in both biological and model membranes [41–43]. These areas, often denominated as 'lipid rafts' [43, 44], are thought to be fundamental in the preservation of the structure and function of the membrane. However, describing the spatiotemporal resolution of these domains has turned out to be a difficult task [43, 45]. It has been suggested that these formations be membrane domains in which signaling from a neurotransmitter may arise via a group of receptors, such as serotonin$_{1A}$ (5-HT$_{1A}$) receptor [46].

Previous studies demonstrated the imperative necessity of membrane cholesterol in the function and organization of the 5-HT$_{1A}$ receptor [45, 47–52]. Results from additional studies showed that the fluidity of lipids considerably regulates the binding of serotonin (5-HT) in murine brain membranes. It is therefore expected that decreased levels of cholesterol would increase the fluidity of the cellular membrane. While, at the same time, minimal exposure of the 5-HT receptors would be found in the synaptic cleft [2, 53].

Reportedly, disturbance of rafts by cholesterol deficiency notably lowers agonist binding and coupling of G protein to 5-hydroxytryptamine 1A (5-HT1A) serotonin receptors in bovine hippocampal membranes [46, 47]. Serotonin$_{1A}$ receptors typify one of the most formidable, evolutionarily primitive, yet largely conserved families of seven transmembrane *G* protein-coupled receptors (GPCRs) that span the membrane [45, 54]. Also, serotonergic signaling constitutes an important part in the formation and regulation of a multitude of functions such as behavioral, cognitive, and developmental [45]. Moreover, studies have demonstrated that there is an association between decreased 5-HT activity and suicide [2, 55].

It is noteworthy to mention that recent studies described crystal structures of GPCRs, including serotonin$_{1A}$ receptor, that demonstrated structural proof of cholesterol binding sites [45, 56, 57].Currently, two conceivable pathways have been proposed by which membrane cholesterol could affect the structure and function of GPCRs: (i) by way of a direct/specific interaction with GPCRs, or (ii) via an indirect pathway by modifying the physical properties of the membrane in which the receptor is inserted, or as a result of an integration of both [45, 58].

About cholesterol levels and their relation to gender, our study showed that the decrease in total cholesterol levels occurred in both men and women. Other authors have reported a relationship between reduced cholesterol and suicidal tendencies only in males [13, 59–62]. However, it is worth noting that additional studies on the association between gender and serum cholesterol have been unconvincing.

A lack of consistency between different published reports coupled with the fact that, to date, it has not been possible to identify a cholesterol threshold level capable of precipitating a psychiatric disorder, suggests the presence of a non-linear relationship.

The existence of reports in which depression has been associated with increased cholesterol levels would support this hypothesis. A possible explanation for this, proposed the involvement of monoamine oxidase (MAO). The aforementioned model studies associated hypercholesterolemia with depression in hypercholesterolemic mice via monoaminergic metabolism. Specifically, they reported increased monoamine oxidase (MAO) A and B activity in the hippocampus of mice [63, 64]. Thus providing one possible reason why elevated levels of cholesterol are able to produce depression much like decreased levels are able to, but via independent mechanisms.

Besides total cholesterol, other studies that investigated the link between triglycerides, HDL cholesterol and LDL cholesterol, observed contrasting results between different populations. Our results showed higher levels of triglycerides in subjects with MDD and MDD associated with suicide attempt. These findings do not coincide with data previously reported in 2015 that observed decreased levels of triglycerides in subjects with suicidal attempts [65]. However, other authors report conflicting findings, even suggesting a positive association between triglyceride levels and the risk of suicidal behavior [15]. Considering LDL cholesterol levels, our results coincide with the conclusions of a meta-analysis published in 2016, which included a total of 36 different studies and found overall association between lower LDL levels and depression [66]. With respect to VLDL levels, which were significantly higher in MDD versus healthy controls, it is worth noting that few studies report VLDL levels. Taking this into consideration, our result is different from the result reported in a previous study [67] that showed significantly lower levels while our results demonstrate higher levels. With respect to HDL cholesterol levels, our results did not demonstrate significant differences when comparing subjects with MDD, subjects with MDD associated with suicide attempt and the control group. This fact contrasts with previous studies in which significant differences in HDL cholesterol between subjects with attempted suicide and healthy controls were shown [59, 68].

Lastly, we found a significant association between elevated triglyceride levels versus MDD and MDD with suicide attempt. Our finding coincides with a previous study which found a correlation between depressive symptoms and triglyceride levels [69] and suggestions by others which postulate that high triglyceride levels are associated with Type A personality traits, such as hostility, anger and domineering attitudes [70].

Several limitations of this study deserve to be mentioned. First, because our study is based on a case-control design, temporality could be not inferred with certainty. Whether hypocholesterolemia is a risk factor for developing depression and suicide attempt or merely an associated epiphenomenon can not be assured with certainty; second, we did not measure 24 S-hydroxycholesterol levels, which is a peripheral biomarker of brain cholesterol metabolism. However, it is expected that reduction of total cholesterol would reduce 24 S-hydroxycholesterol [71]. Besides, we did not evaluate dietary intake; however, because subjects in the groups of the study were enrolled from the same socio-cultural and economic background, it is expected that customary diets were similarly distributed. Finally, an additional limitation of our study is that we did not analyze the cardiovascular risk associated with serum cholesterol concentrations. In the future, it will be important to profoundly analyze the contradictory results reported with regard to cholesterol's role in depression. [63, 64]. This would help to verify if cholesterol is, in fact, a viable biomarker for neuropsychiatric disorders. It is evident that these results may be extrapolated only to a population that is similar to our own with similar exclusion criteria. Strengths of our study include the inclusion of incident cases of suicide attempt, which is a recognized tool to minimize analysis bias in the cross-sectional studies; also, the exclusion of individuals with lipid-lowering drugs allows us to control the potential source of bias.

Conclusions

In conclusion, our results show that hypocholesterolemia is independently associated with depression and suicide attempt in adults of the Mexican population .This finding, if consistent in more studies in our population, could influence public health policies focused in the prevention of mental health disorders. Measuring cholesterol levels could be a minimally invasive, inexpensive and simple way to predict suicide risk in our population. The use of cholesterol levels as a biomarker would permit clinicians to efficiently obtain a laboratory result that, once combined with clinical evaluations and symptoms, could permit a more timely diagnosis. Additionally, it would be necessary to calculate the sensibility and specificity of this test in our population.

Abbreviations

CHOL: Cholesterol; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; MDD: Major depressive disorder; VLDL: Very low-density lipoprotein

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
EMH and FCJ conceived and designed the experiments; MCC, EMM and MSM performed the experiments; OLL and JSP analyzed the data; OAC and MBS contributed analysis tools; FVA, JMH and EMM wrote the paper. All authors read and approved the final manuscript

Ethics approval and consent to participate
The protocol was approved by the Ethics Committee for Research of the General Hospital 450, Secretary of Health of Durango (No. 12/0306-2015). Informed consent was obtained from participants was in written form.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests

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Auditory- and Vestibular-Evoked Potentials Correlate with Motor and Non-Motor Features of Parkinson's Disease

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Degeneration of several brainstem nuclei has been long related to motor and non-motor symptoms (NMSs) of Parkinson's disease (PD). Nevertheless, due to technical issues, there are only a few studies that correlate that association. Brainstem auditory-evoked potential (BAEP) and vestibular-evoked myogenic potential (VEMP) responses represent a valuable tool for brainstem assessment. Here, we investigated the abnormalities of BAEPs, ocular VEMPs (oVEMPs), and cervical VEMPs (cVEMPs) in patients with PD and its correlation to the motor and NMSs. Fifteen patients diagnosed as idiopathic PD were evaluated by Unified Parkinson's Disease Rating Scale and its subscores, Hoehn and Yahr scale, Schwab and England scale, and Non-Motor Symptoms Scale. PD patients underwent pure-tone, speech audiometry, tympanometry, BAEP, oVEMPs, and cVEMPs, and compared to 15 age-matched control subjects. PD subjects showed abnormal BAEP wave morphology, prolonged absolute latencies of wave V and I–V interpeak latencies. Absent responses were the marked abnormality seen in oVEMP. Prolonged latencies with reduced amplitudes were seen in cVEMP responses. Rigidity and bradykinesia were correlated to the BAEP and cVEMP responses contralateral to the clinically more affected side. Contralateral and ipsilateral cVEMPs were significantly correlated to sleep ($p = 0.03$ and 0.001), perception ($p = 0.03$), memory/cognition ($p = 0.025$), and urinary scores ($p = 0.03$). The oVEMP responses showed significant correlations to cardiovascular ($p = 0.01$) and sexual dysfunctions ($p = 0.013$). PD is associated with BAEP and VEMP abnormalities that are correlated to the motor and some non-motor clinical characteristics. These abnormalities could be considered as potential electrophysiological biomarkers for brainstem dysfunction and its associated motor and non-motor features.

Keywords: Parkinson's disease, motor, non-motor, vestibular, auditory, evoked potentials

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder caused by degeneration of midbrain dopaminergic neurons of substantia nigra (SN) producing its primary motor cardinal features (1). In addition to the motor symptoms, PD patients develop a variety of non-motor symptoms (NMSs), which significantly impair their quality of life. The NMSs consist of autonomic dysfunction, sensory symptoms, neuropsychiatric disturbances, sleep disorders, fatigue, and gastrointestinal disorders (2). Those NMSs are associated with dopaminergic and non-dopaminergic dysfunctions including serotoninergic, noradrenergic, and cholinergic systems (3, 4). Degeneration of several brainstem nuclei and their connections is responsible—at least partially—for different neurotransmitters disruption, resulting in different NMSs (3, 5).

Lewy bodies (LB) and Lewy neurites (LN) composed of alpha-synuclein are the pathological hallmarks of PD (6, 7). Alpha-synuclein pathology related to NMS was described in brain hemispheres, brainstem, spinal cord, and peripheral nervous system (5). Recent studies emphasized the importance of brainstem as the habitat of degeneration of nuclei responsible for different NMSs (8). Moreover, some NMS-related pathological changes might have specific distribution and anatomical localization in various levels of the brainstem. For example, depression and REM sleep behavior disorder are related to degeneration of pontine nuclei (5).

These reports are consistent with Braak's proposal of pathological progression of PD, which starts caudally from the dorsal motor vagal nucleus in the medulla and then ascends in the brainstem and finally involves neocortex (9, 10). Thus, most brainstem nuclei are involved in early stages (I–III) that explain the preclinical and early emergence of NMS, while SN is involved in stage III (3, 5). Recently, Seidel et al. demonstrated the widespread of LB and LN in brainstem nuclei and fiber tracts including vestibular nuclei (4). Therefore, diagnostic tools exploring disruption of lower brainstem nuclei and related NMS are needed for early diagnosis of PD (11).

Brainstem auditory-evoked potential (BAEP) and vestibular-evoked myogenic potential (VEMP) responses represent a valuable tool for brainstem assessment as the neural pathways of both ocular VEMPs (oVEMPs) and cervical VEMPs (cVEMPs) pass through the brainstem (12). VEMPs are short latency manifestations of vestibulo-ocular reflex connecting VIII and III cranial nuclei and vestibulo-collic reflex connecting VIII and XI cranial nuclei that originate from the utricle and saccule, respectively (12). Previous studies described impaired BAEP and VEMP responses in PD patient compared to controls that were attributed to underlying brainstem dysfunction (13–16). Furthermore, impairment of these responses was related topographically to other brainstem lesions. Principally, the BAEP and oVEMP responses are affected in upper brainstem (midbrain) lesions, while cVEMP responses are involved in the lower brainstem (pontine and upper medullary) lesions (17–19). Therefore, they have a localizing value of brainstem dysfunction at different levels.

In the current study, we hypothesized that NMSs of PD related to brainstem dysfunction could be related to changes in VEMPs and BAEP. Accordingly, the aim of this study was to explore the abnormalities of BAEPs and VEMPs in patients with PD and its correlation to the motor and NMSs of PD.

MATERIALS AND METHODS

Materials

Fifteen patients diagnosed as idiopathic PD and 15 age-matched control subjects were included in the current prospective study. PD patients were recruited from the movement disorders outpatient clinic at Ain Shams University Hospitals, Cairo, Egypt in the period between 2013 and 2015. Recruited patients were diagnosed as idiopathic PD according to the British Parkinson's Disease Society Brain Bank criteria (20). Exclusion criteria included dementia (MMSE score <24), improper neck movements that interfere with audiological assessment, middle ear diseases, and hearing thresholds exceeding 50 dBnHL.

All subjects were evaluated using Unified Parkinson's Disease Rating Scale (UPDRS), Hoehn and Yahr scale (H&Y), and Schwab and England scale (S&E) in "medication off" and "on" states by a movement disorders expert. Different UPDRS subscales were estimated including the activity of daily living (UPDRS I), a motor (UPDRS III), UPDRS I, and total UPDRS scores. Furthermore, main motor symptoms subscores were calculated such as tremor (items 20 and 21 of UPDRS), rigidity (item 22), bradykinesia (items 18, 19, 23, and 24), axial signs (items 27, 28, 29, and 30), postural instability/gait disability score (items 13, 14, 15, 29, and 30) (21). The NMSs were measured for all patients by the Non-Motor Symptoms Scale (NMSS) administrated by the movement disorders expert (2). Control subjects were age- and sex-matched normal volunteers and provided a reference of auditory and vestibular work up. Informed consent was taken from all subjects before participation in the present study. The study protocol was approved by the Ethics Committee of Faculty of Medicine, Ain Shams University. All subjects gave written informed consent by the Declaration of Helsinki.

Procedures

Audio-Vestibular Works Up

Basic audiological evaluation to assess the peripheral auditory system, pure-tone (PTA), and speech audiometry was done using the two-channel audiometer Grason-Stadler Inc. (GSI, Eden Prairie, MN, USA) model 61 calibrated according to ANSI (1969) in a sound-treated room IAC model 1602 (IAC Acoustics, UK). The middle ear functions were tested through the acoustic immittance meter Grason-Stadler Inc. (GSI, Eden Prairie, MN, USA) model 33. BAEP and VEMP were done to all subjects of the study using the ICS Chartr EP 200—GN Otometrics (Denmark)-evoked potential system.

For the BAEP assessment, the active electrode was mounted to the middle of the forehead "Fpz," the reference electrode to the ipsilateral mastoid "M1," and the ground to the contralateral one "M2." The test procedures followed Sininger protocol (22). Analysis of BAEP was done quantitatively to assess the absolute latencies of waves I, III, and V and interpeak latencies of these waves (I–III, III–V, and I–V). This was done both at high stimulus level "90 dBnHL" and at lower intensities down to thresholds. The

interaural latency difference and the latency/rate function were studied at high stimulus intensity. Qualitative analysis of the waveform morphology comprised the subjective judgment on the shape and the quality of the waveforms.

Vestibular-Evoked Myogenic Potentials

oVEMP Test

Monaural stimulation with contralateral eye recording was employed for recording oVEMPs (23). Three surface electromyography (EMG) electrodes were placed on the face just inferior and at the center of lower eyelid (the active electrode), the chin (the reference electrode), and the forehead (the ground electrode). During recording, all subjects were instructed to look upward at a small fixed target >2 m from the eyes, while the vertical eye position was at an angle of approximately 30–35° above horizontal. The oVEMP response included the initial negative–positive biphasic waveform comprised peaks n1 and p1. Two runs were performed for each test to confirm the reproducibility of results. The latencies of peaks n1 and p1, amplitude n1–p1, and interaural amplitude difference (IAD) ratio were measured.

cVEMPs Test

The subject was seated with the head rotating sideways toward one shoulder to activate the sternocleidomastoid (SCM) muscle. The active electrode was placed at midpoints of each SCM muscle on symmetrical sites, the reference electrode on the suprasternal notch, and the ground electrode on the forehead. Monaural acoustic stimulation with ipsilateral recording was employed for recording cVEMPs. The n13–p13 wave latencies, amplitudes, and IAD were measured (24).

Assessment of auditory and vestibular responses was done during medications "On" states to decrease EMG artifacts and ensure patients cooperation. The findings of BAEPs, cVEMPs, and oVEMPs of PD patients were grouped to ipsilateral and contralateral to the clinically more affected (CMA) side and compared to the mean of both sides of the control subjects, then VEMP responses are correlated to different UPDRS Off-scores and NMS scores. **Figure 1** shows samples of cVEMP and oVEMP responses of one of the recruited subjects.

Statistical Analysis

Statistical analyses were performed using the SPSS version 18. Qualitative data were described using number and percent, while quantitative data were described using mean and SD. Association between categorical variables was tested using chi-square test. Comparison between two independent variables was done using independent *t*-test. Correlations between quantitative variables were assessed using Spearman coefficient. The level of statistical significance (*p* value) was set at 0.05 (significant) and 0.01 (highly significant).

RESULTS

Fifteen patients with idiopathic PD (12 males and 3 females) completed the clinical and audiological assessments. Their mean age was 59.20 ± 10.08 years (ranged from 35 to 70 years), and the duration of illness was 5.50 ± 2.96 years (2–10 years). The mean total UPDRS and mean disease disability (S&E scale) scores were 41.33 ± 30.20 and 68.67 ± 22.30, respectively. Patients were of variable disease stages ranged from stage 2 to 5 of H&Y scale in "Off" state with mean 2.73 ± 0.84. All patients had at least impaired domain of NMS. Sleep/fatigue (86.7%) and mood/cognition (73.3%) were the most frequent reported NMS. Detailed motor and non-motor scores of the PD patients are presented in **Tables 1** and **2**.

Audiological Work Up

The PD patients had significantly higher PTA thresholds mainly in the high frequencies 4 and 8 kHz bilaterally compared to the age-matched control group ($p < 0.03$). Seven PD subjects (46.7%) had sensorineural hearing loss of mild to moderate degree (bilateral symmetrical in five and unilateral in two).

Brainstem Auditory-Evoked Potentials

The ipsilateral and contralateral absolute latencies of wave V ($p = 0.04$) and I–V interpeak ($p = 0.025$ and 0.03) latencies were significantly prolonged compared to controls. Moreover, the wave III and the interpeak I–III latencies were also significantly prolonged ($p = 0.03$ and 0.036, respectively) ipsilateral to the

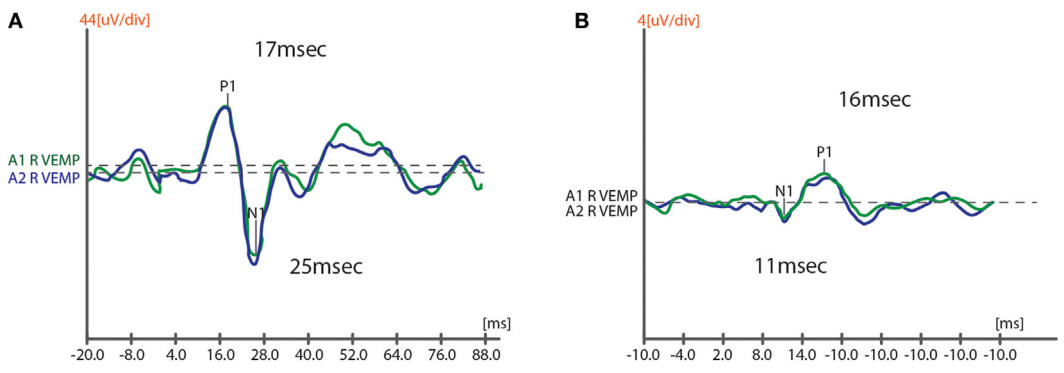

FIGURE 1 | A sample of recorded response of cVEMP (A) and oVEMP (B) in one of study subjects. cVEMPs, cervical vestibular-evoked myogenic potentials; oVEMP, ocular VEMPs.

TABLE 1 | Demographic and clinical characters of Parkinson's disease patients.

Clinical feature	Mean ± SD (range)
Age (years)	59.20 ± 10.08 (35–76)
Duration of disease (years)	5.50 ± 2.96 (2–10)
Age of onset (years)	53.77 ± 11.49 (25–70)
H&Y off	2.73 ± 0.84 (2–5)
H&Y on	0.93 ± 0.59 (0–2)
S&E off	68.67 ± 22.30 (50–90)
S&E on	90.0 ± 9.26 (70–100)
UPDRS I off	3.33 ± 2.13 (0–6)
UPDRS II off	12.73 ± 7.49 (0–27)
UPDRS III off	30.20 ± 17.49 (2–69)
UPDRS IV	0.67 ± 1.40 (0–4)
UPDRS-total	41.33 ± 30.20 (2–109)
Postural instability/gait disability off	5.20 ± 4.06 (0–16)
Axial off	5.27 ± 4.28 (0–15)

H&Y, Hoehn and Yahr scale; S&E, Schwab and England scale; UPDRS, Unified Parkinson's Disease Rating Scale.

TABLE 2 | Severity and prevalence of non-motor manifestations of Parkinson's disease patients.

NMS	Mean ± SD (range)	Prevalence (%)
NMS CVS	0.93 ± 1.67 (0–6)	40
NMS sleep/fatigue	7.07 ± 6.36 (0–22)	86.7
NMS mood and cognition	11.60 ± 12.54 (0–42)	73.3
NMS perception/hallucinations	0.80 ± 1.27 (0–4)	33.3
NMS memory	5.00 ± 6.07 (0–20)	60
NMS GIT	2.80 ± 3.78 (0–9)	53.3
NMS urinary	5.87 ± 8.96 (0–33)	60
NMS sexual	3.93 ± 5.81 (0–16)	46.7
NMS miscellaneous	3.33 ± 4.67 (0–17)	60
NMS total	41.33 ± 30.20 (2–109)	100

NMS, non-motor symptoms; CVS, cardiovascular system; GIT, gastrointestinal tract.

CMA side. Eight patients (53%) had an abnormal BAEP wave morphology.

Ocular VEMPs

Absent oVEMP responses were the commonest abnormality and were detected in 47% of PD subjects ($n = 7$). Compared to control group, the latencies of n1 and p1 were significantly prolonged when contralateral to the CMA side ($p = 0.04$ and 0.025), and n1–p1 amplitude was significantly reduced bilaterally ($p < 0.001$).

Cervical VEMPs

The cVEMP responses were absent in three patients (20%). Compared to controls, the ipsi- and contralateral p13, and contralateral n23 latencies were significantly prolonged ($p = 0.04$, 0.001, and 0.04, respectively) and bilateral p13–n23 amplitudes were significantly decreased ($p < 0.001$).

Correlations between BAEP, VEMPs, and UPDRS Scores

The contralateral absolute latencies of waves III and V were significantly correlated to disease severity (H&Y scale) ($r = 0.610$, $p = 0.028$ and $r = 0.530$, $p = 0.043$, respectively) and wave V with rigidity "Off" score ($r = 0.540$, $p = 0.039$). Furthermore, the absence of BAEP waves contralateral to the CMA was significantly correlated to S&E, UPDRS III, and rigidity "Off" scores ($r = 0.665$, $p = 0.007$; $r = -0.540$, $p = 0.037$; and $r = -0.770$, $p = 0.001$, respectively).

The abnormal cVEMP responses contralateral to the CMA side showed significant correlation to "H&Y" disease stage (wave latency) ($r = 0.689$, $p = 0.013$), UPDRS III ($r = 0.523$, $p = 0.045$), rigidity (wave latency) ($r = 0.634$, $p = 0.027$), and bradykinesia "Off" scores (wave absence) ($r = 0.571$, $p = 0.026$). The ipsilateral p13 and n23 wave latencies were also correlated to dyskinesia scores ($r = 709$, $p = 0.01$ and $r = 634$, $p = 0.027$, respectively). Furthermore, the oVEMP responses ipsilateral to the CMA side showed moderate correlation with a trend to significance with UPDRS III, rigidity, and axial "off" scores ($p = 0.046$, 0.05, and 0.049, respectively).

On the other hand, the UPDRS II, tremor, and S&E subscales showed no significant correlations with BAEP and VEMP responses. The duration of the disease, age, and age of onset showed no correlation either with BAEP, cVEMP, and oVEMP responses.

Correlations between BAEP, VEMPs, and Non-Motor Scores

The BAEP showed minor associations to some non-motor functions scores. The contralateral I–III and I–V interpeak latencies were correlated to NMS-gastrointestinal tract (GIT) scores ($r = 0.625$, $p = 0.03$ and $r = 0.595$, $p = 0.041$, respectively), while the ipsilateral I–III interpeak latency was correlated to sleep/fatigue scores ($r = 0.586$ and $p = 0.035$).

Contralateral and ipsilateral cVEMP responses showed significant correlations to most of NMSS domains. They were significantly correlated to mood/cognition (0.024), sleep/fatigue ($p = 0.03$ and 0.001), perception ($p = 0.03$), memory ($p = 0.025$), and urinary scores ($p = 0.03$). Sexual dysfunction was moderately correlated to the absence of ipsilateral responses ($p = 0.045$). The oVEMP responses showed significant correlations to fewer NMS domains including NMS cardiovascular system ($p = 0.01$), sexual dysfunction ($p = 0.013$), and perception (moderate correlation, $p = 0.047$) (see **Table 3**; **Figure 2**). GIT domain showed no significant correlation with VEMP responses.

DISCUSSION

The current study investigated comprehensively the brainstem dysfunction of PD patients and its relation to motor and NMSs using BAEPs and VEMPs. It confirmed the impairment of BAEP and VEMP responses in patients with PD compared to controls that are related to clinical asymmetry of PD and its cardinal motor features. The BAEPs and cVEMPs were correlated significantly to contralateral motor UPDRS, rigidity, and bradykinesia severity, and ipsilateral dyskinesia scores. Moreover, cVEMP responses were correlated to some non-motor features including sleep/fatigue, mood/cognition, perception, and memory. BAEP and oVEMP responses showed less correlation to the motor and

non-motor features. Consequently, it reflects brainstem pathology among PD patients at different levels and highlights the asymmetry of these changes.

TABLE 3 | Positive correlation between NMS scores and VEMP responses.

NMS	Related ARB and vestibular-evoked myogenic potential (VEMP) responses	*r*	*p*
CVS	Contralat ocular VEMPs (oVEMP) absent response	−0.643	0.01
Sleep/fatigue	Ipsilat I–III	0.586	0.035
	Contralat cervical VEMPs (cVEMPs) n23 latency	0.845	0.001
	Contralat cVEMP p13 latency	0.583	0.047
	Ipsilat cVEMP pn amplitude	−0.625	0.03
GIT	Contralat I–III latency	0.625	0.03
	Contralat I–V latency	0.595	0.041
Mood/cognition	Contralat cVEMP n23 latency	0.643	0.024
Perception/ hallucination	Ipsilat cVEMP pn amplitude	−0.625	0.03
	Contralat oVEMP p1 latency	0.674	0.047
Memory	Contralat cVEMP pn amplitude	−0.639	0.025
Urinary	Ipsilat cVEMP n23 latency	0.622	0.031
Sexual	Contralat oVEMP n1 latency	0.782	0.013
	Ipsilat cVEMP absent response	0.524	0.045

NMS, non-motor symptoms; CVS, cardiovascular system; GIT, gastrointestinal tract; Ipsilat, ipsilateral; contralat, contralateral (to clinically more affected side).

In this study, we separated the vestibular responses to ipsilateral and contralateral responses according to CMA side. This approach could reflect the expected asymmetrical brainstem pathology of PD (25). Previous studies confirmed the asymmetry of cardinal motor symptoms that often persists through the course of the disease. This clinical asymmetry is related to asymmetrical degeneration of dopaminergic neurons of SN, striatal dopaminergic receptors, and their cortical connections (8, 25). Furthermore, the clinical asymmetry could be related to NMSs including pain, fatigue, depression, and cognitive dysfunction. This could be explained by asymmetrical underlying pathological changes and the asymmetrical dopaminergic deficiency that contributes to some non-motor features along with other neurotransmitters (8, 25).

The high-frequency hearing impairment associated with PD was detected in the current work similar to prior studies (26–28). Impaired hearing in PD was attributed to peripheral auditory dysfunction (27, 28) and abnormal central auditory processing (29, 30). Likewise, abnormal BAEPs among PD patients were demonstrated inconsistent to previous studies, denoting brainstem auditory conduction delay (26, 31, 32).

Additionally, both the oVEMPs and cVEMPs were affected in the studied PD subjects. The findings of the present study agreed with de Natale et al. who reported that the frequency of alteration of VEMPs in PD patients was 83.3% when considering the

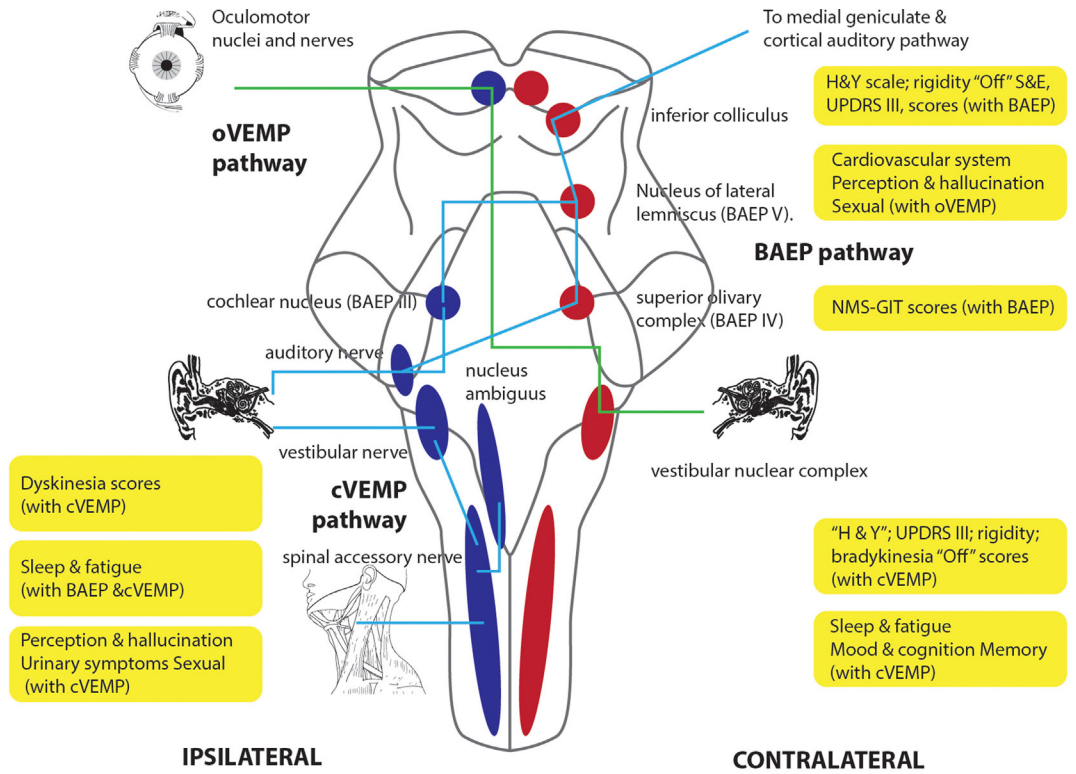

FIGURE 2 | The schematic diagram describes the topographic distribution of different motor and non-motor manifestations of Parkinson's disease patients according to its correlation with BAEP, cVEMP, and oVEMP abnormal responses in the current study. BAEPs, brainstem auditory-evoked potentials; cVEMPs, cervical vestibular-evoked myogenic potentials; oVEMP, ocular VEMPs.

combined set of cVEMP, oVEMP, and masseter VEMP responses, with absence being the prevalent alteration in PD (14). Similarly, Pollak et al. showed unilaterally absent VEMP responses in 20 (37%) of PD patients and bilaterally absent responses in 4 patients (7.4%) (33). Lower amplitudes of cVEMPs among PD patients were reported by another study (34). However, a recent study by Pötter-Nerger et al. reported abnormal oVEMPs in mild to moderate PD patients with preserved cVEMPs that were attributed to methodological differences (16). These findings emphasize the extensive brainstem dysfunctions at different anatomical levels (35).

Auditory and vestibular dysfunctions in PD could be explained by different mechanisms. Modulatory effect of dopamine on the excitability of vestibular nuclei is one of these mechanisms (34). Disrupted interconnections of vestibular nuclei with degenerated other brainstem nuclei by PD pathology especially dorsal raphe nuclei is another mechanism (36, 37). Furthermore, direct disruption of vestibular nuclei by PD pathological changes produces these vestibular abnormalities as recently reported (4).

Remarkably, the current study defined correlation of the main motor features of PD with BAEPs and cVEMPs rather than oVEMPs that might be explained by the midbrain and pontine pathological changes and non-involvement of vestibulo-ocular pathways in the pathophysiology of these features. Rigidity and bradykinesia were related to BAEP and cVEMP responses, while tremor was not. The correlations were mainly to the responses contralateral to CMA side, which is consistent with asymmetric nature of PD pathology in SN and their connections (25).

Tremor has different pathophysiology compared to rigidity and bradykinesia and characterized by involvement of the cerebellum–thalamocortical circuit in its pathogenesis (37). This explains the lack of correlation with auditory and vestibular responses. Moreover, recent animal studies demonstrated that brainstem structures such as pontine nuclei and locus coeruleus are involved in the pathophysiology of levodopa-induced dyskinesia (LID) (38). This could explain the correlation seen in the present study between LID and cVEMP wave latencies.

Although the correlation of BAEPs, oVEMPs, and cVEMPs were mainly to one side, yet no differences existed in all tests between the two sides. The medication state of the subjects could be the explanation. All PD subjects underwent the tests during "medication on state" that masked the abnormalities between the two sides as recently reported by Pötter-Nerger et al. (34).

In contrast, previous studies reported a lack of correlation with clinical motor scores (14, 33, 34). Nonetheless, de Natal et al. defined the progression of VEMP abnormalities with increased stage of the disease (14). They used the mean values of VEMP responses on both sides, not about the CMA side, thus underestimating the potential asymmetry that could ameliorate abnormalities. Moreover, differences in experimental conditions during testing, age differences between cases and controls, and different clinical characteristics of recruited patients could explain the inconsistency of results of different studies that addressed the vestibular functions in PD.

Few prior studies investigated the correlation of auditory and vestibular responses with individual NMSs of PD (14, 33). In the current study, sleep and mood domains' severity demonstrated correlation with cVEMP responses that could be related to associated dysfunction of different pontine nuclei such as locus coeruleus, raphe nucleus, and pedunculopontine nucleus (3). Previous studies reported localizing function of cVEMP responses of pontine lesions associated with different other diseases (17–19). Recently, de Natale et al. found a direct correlation between VEMP changes and REM sleep behavior disorder and postural instability (14). The same study reported a lack of correlation with depression (14), while a study by Pollack et al. reported a correlation of cVEMP with depression and antidepressants use (33).

Unsurprisingly, GIT domain that includes hypersalivation, dysphagia, and constipation had minor correlations. This could be attributed to the high contribution of peripheral pathological changes in GIT and related nerve supply (alpha-synucleinopathy) along with brainstem changes (39–41). Furthermore, GIT symptoms are attributed to dysfunction of the dorsal motor nucleus of the vagus, nucleus ambiguous, and nucleus of a solitary tract located in the medulla (3) that is poorly localized by VEMP responses (13). Correlation of GIT symptoms' severity to contralateral BAEP latency could be explained by associated advanced disease (42). Similarly, the contribution of sacral spinal cord alpha-synuclein deposition to urinary symptoms explains limited correlation to vestibular responses (43). Likewise, correlation of other non-motor features poorly linked to brainstem dysfunction such as cardiovascular symptoms, attention/memory, and perception/hallucination could be explained by associated disease severity (42). Cognitive deficits of PD are correlated to higher order auditory processing in subcortical–cortical pathways evaluated by event-related potentials (P300) (29, 44).

The current study has different limitations. These limitations include a small number of recruited sample, including patients with different disease severity and stages, and the use of NMSS subscores that describe different NMSs for correlations with vestibular responses. Thus, further, more specific tools for each NMS are required for correlation with the electrophysiological assessment of the larger number of PD patients.

In conclusion, the current study confirms the auditory and vestibular abnormalities among PD patients that reflect brainstem pathological changes. It also correlates these abnormalities to some motor and non-motor features of the disease, providing a localizing tool for associated brainstem dysfunction. Furthermore, abnormal vestibular potentials are related to disease severity and stage and might respect clinical and pathological asymmetry. However, further studies are warranted to reproduce the correlation of VEMP responses to the individual motor and NMSs of PD and their value as potential biomarkers at different stages of the disease, especially the early PD.

ETHICS STATEMENT

The ethical committee of the Faculty of Medicine, Ain Shams University has allowed doing this study. The authors assert that all procedures contributing to this work comply with the ethical

standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

AUTHOR CONTRIBUTIONS

AS and DH: research idea and conception, data acquisition, data analysis and interpretation, and manuscript writing and reviewing. HE, EM-H, and JS-P: reviewing the manuscript. MS: data analysis and manuscript reviewing. OA-C: data output analysis and manuscript writing and reviewing.

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Article

Effects of *Moringa oleifera* Leaves Extract on High Glucose-Induced Metabolic Changes in HepG2 Cells

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Abstract: Mitochondrial dysfunction is a hallmark of diabetes, but the metabolic alterations during early stages of the disease remain unknown. The ability of liver cells to rearrange their metabolism plays an important role in compensating the energy shortage and may provide cell survival. *Moringa oleifera* leaves have been studied for its health properties against diabetes, insulin resistance, and non-alcoholic liver disease. We postulated that *M. oleifera* executes a protective function on mitochondrial functionality in HepG2 treated with high glucose. We evaluated the effect of high glucose treatment on the mitochondrial function of HepG2 cells using a Seahorse extracellular flux analyzer (Agilent, Santa Clara, CA, USA), blue native polyacrylamide gel electrophoresis (BN-PAGE), and western blot analysis. For assessment of mitochondrial abnormalities, we measured the activity of mitochondrial Complex I and IV as well as uncoupling protein 2, and sirtuin 3 protein contents. Our results demonstrate that, under conditions mimicking the hyperglycemia, Complex I activity, UCP2, Complex III and IV subunits content, supercomplex formation, and acetylation levels are modified with respect to the control condition. However, basal oxygen consumption rate was not affected and mitochondrial reactive oxygen species production remained unchanged in all groups. Treatment of HepG2 cells with *M. oleifera* extract significantly increased both protein content and mitochondrial complexes activities. Nonetheless, control cells' respiratory control ratio (RCR) was 4.37 compared to high glucose treated cells' RCR of 15.3, and glucose plus *M. oleifera* treated cells' RCR of 5.2, this indicates high-quality mitochondria and efficient oxidative phosphorylation coupling. Additionally, the state app was not altered between different treatments, suggesting no alteration in respiratory fluxes. These findings enhance understanding of the actions of *M. oleifera* and suggest that the known antidiabetic property of this plant, at least in part, is mediated through modulating the mitochondrial respiratory chain.

Keywords: HepG2 cells; *Moringa oleifera*; mitochondria; UCP2; SIRT3

1. Introduction

A growing body of experimental and epidemiological evidence suggests that *Moringa oleifera* Lam have antidiabetic and antioxidant effects against the harmful damages of oxidative stress and diabetic complications [1–3]. The beneficial activities of *M. oleifera* on carbohydrate metabolism have been shown by different physiological processes, including preventing and restoring the integrity and function of pancreatic β-cells, increasing insulin action, improving glucose uptake and utilization [4]. Recent studies have demonstrated that phenolic compounds of *M. oleifera* significantly decreased total intracellular cholesterol, inhibited the activity of HMG CoA reductase (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase), and enhanced low-Density Lipoprotein (LDL) receptor binding activity in HepG2 cells [5]. Furthermore, it was reported that the *M. oleifera* during adipogenesis improves adipocyte functionality and upregulates the expression of uncoupling protein 1 (UCP1), sirtuin 1 (SIRT-1), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) involved in thermogenesis modulating lipid metabolism [6]. In association with these results, it has been suggested that mitochondrial proton leak (UCP activity) might play a role in the pathophysiology of diabetic complications [7] and cardiovascular diseases [8]. We recently demonstrated changes in oxygen consumption, supercomplex formation, and increased lipoperoxidation levels in isolated mitochondria from liver of streptozotocin (STZ)-diabetic rats, where *M. oleifera* extract may have a protective role against some of these alteration [9]. These data suggested that *M. oleifera* works intracellularly via several metabolic pathways within mitochondria. Thereby, there are diverse promising candidates for suppressing mitochondrial dysfunction, two potential targets that caught our attention are sirtuins (SIRTs) and uncoupling proteins (UCPs) which seem to be critically important in the pathogenesis of diabetes, cardiovascular disease, and obesity [10].

The SIRTs are a family of nicotinamide adenine dinucleotide (NAD^+) dependent deacetylases and they play a critical role in restoring homeostasis during stress responses. They can influence multiple protein functions, including DNA—protein interactions, transcriptional activity, subcellular localization, protein stability, and enzymatic activity [11,12]. The crucial role played by SIRTs in the regulation of metabolism has been extensively studied. For example, in liver and heart, SIRT1 regulates gluconeogenic activity by modulating cAMP responsive element binding protein, PGC-1α a nuclear-encoded transcriptional coactivator that regulates the expression of nuclear-encoded mitochondrial proteins, including nuclear respiratory factors 1 and 2 (NRF1 and NRF2), estrogen-related receptor-α (ERR-α), and mitochondrial transcription factor A (TFAM) [13,14]. In addition, SIRT1 activators improve insulin sensitivity in liver and heart. SIRT3 is a member of the sirtuin family that is localized in mitochondria. It is decreased in skeletal muscle of diabetic models [15] and it has been shown that high fat feeding induces a shift in acetylation balance, causing protein hyperacetylation in liver [16], SIRT3 is also involved in the regulation of oxidative phosphorylation through the deacetylation of Complex I and succinate dehydrogenase subunits [17,18]. In diabetes, increases in mitochondrial uncoupling induce mild metabolic stress by dissipating the hydrogen ion gradient across the inner mitochondrial membrane. As a stress response, glucose uptake and NADH oxidation are stimulated with an increase in respiration, NAD^+ levels, and the NAD^+/NADH ratio in mitochondria. Thus NAD^+ dependent SIRT1 is activated [19]. Therefore, SIRT1 and 3 regulation is a promising new therapeutic approach for treating diabetic complications [20] and some research groups are now focusing on the development of high affinity small molecule activators of SIRT1 [21,22].

In this context, it was demonstrated that resveratrol significantly increases SIRT1 activity through allosteric interaction, resulting in the increase of SIRT1 affinity for both NAD^+ and the acetylated substrate, causing deacetylation of PGC1α, forkhead box protein O1 (FOXO1), and the target of rapamycin kinase 2 (TORC2) which in turn leads to increased fatty acid oxidation and gluconeogenesis [23,24]. However, it is still unknown whether these mitochondrial defects result in change in others SIRTs.

On the other hand, UCPs are a family of carriers expressed in the mitochondrial inner membrane that uncouple oxygen consumption by the respiratory chain from ATP synthesis. UCP2 is expressed in

a wide range of tissues and acts in the protection against oxidative stress, in the negative regulation of insulin secretion by beta cells, and in fatty acid metabolism. Most of the studies about the role of UCP2 in diabetes have focused on the UCP2 functions in β-cells, and the results have shown a deleterious effect of UCP2 in diabetes [25]. As UCP2 is widely expressed in many tissues such as liver, its antioxidant activity makes it logical to search look for benefits on diabetes through counteracting the oxidative stress appeared in diabetes and its complications. Moreover, Korean red ginseng promoted the expression of insulin and downregulated the expression of UCP2 in spontaneously diabetic Goto-Kakizaki rats [26]. Due to the close correlation between these proteins (SIRT and UCP) and mitochondrial oxidative phosphorylation, we hypothesized that *M. oleifera* may exert a protective effect against the development of diabetes through regulatory effect of SIRT3 and UCP2.

Finally, HepG2 cells are a suitable and well characterized model of human liver, which has been widely used in biochemical and nutritional studies [27–29]. Numerous studies have used a high concentration of glucose (25 mM) as an in vitro model for investigation of hyperglycemia-induced toxicity which simulated in vivo condition of diabetic ketoacidosis observed in acute or untreated diabetes [30–32]. Therefore, we used this cell line to explore the effect of high glucose on modulating mitochondrial SIRT3 and UCP2 and the possible protective role of *M. oleifera* over these alterations to determine the molecular targets by which this plant exerts its beneficial properties in the treatment of diabetes.

2. Materials and Methods

2.1. Reagents

All the reagents used in this study were of reagent grade and were purchased from Sigma Aldrich (Toluca, Mexico); Gibco, Thermo Fisher Scientific (Waltham, MA, USA); Invitrogen Thermo Fisher Scientific (Waltham, MA, USA); and Abcam (Cambridge, MA, USA).

2.2. Moringa oleifera Extract Preparation

Extract preparation was carried out as previously described [33]. 100 g of crushed dried moringa leaves were macerated for 24 h in 1 L of 20:80 v/v methanol in constant stirring. Solution was then filtered and distilled in a rotary evaporator at 60 °C, the aqueous fraction was then frozen at −80 °C for 24 h prior freeze drying to yield the final powdered extract. The extract was stored at −80 °C until its use.

2.3. Cell Culture

HepG2 cells were grown in 75 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) (5 mM glucose) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin and kept at 37 °C with 5% CO_2. Media was changed every third day until confluence was reached. Then, cells were divided into three groups: control (C); 25 mM glucose (G) and 25 mM glucose + 500 µg/mL of *M. oleifera* extract (GM). Cells were kept in high glucose media for 24 h. After incubation, cells were washed with fresh DMEM and kept in media supplemented with moringa extract for 2 h. Cells were detached with trypsin and resuspended in DMEM to inactivate trypsin, then centrifuged at 600× *g* for 10 min to recover the cellular pellet.

2.4. Mitochondrial Isolation

Mitochondria from HepG2 cells were isolated with mitochondria isolation kit (MITOISO2 Sigma) following manufacturer indications.

2.5. Viability Assay

Ninety-six-well plates were seeded with 5000 cells per well to perform viability experiments. Cells were incubated with different concentrations of moringa extract (50–500 µg/mL), glucose (50 mM),

and the combination of both for 24 h at 37 °C with 5% CO_2. Cells were then incubated for 2 h with 30 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution per well. The media was discarded and 100 µL of dimethyl sulfoxide (DMSO) was used to dissolve the formazan crystals and to extract the blue color. Absorbance was read in a microplate reader at 595 nm.

2.6. Oxygen Consumption Rate

A measure of 30,000 cells/mL were seeded in 24-well seahorse XF-24 plates and let attach for 24 h. Cells were then incubated with glucose (25 mM) for 24 h, followed by moringa extract addition (500 µg/mL) for 2 h. After incubation, media was replaced with DMEM without FBS and placed at 37 °C without CO_2. The injection ports were loaded with sequence of oligomycin, carbonyl cyanide 4 (trifluoromethoxy) phenylhydrazone (FCCP), rotenone (Rot), and antimycin A (AA) for final assay concentrations of 2.5 µg/mL, 2.5 µM, 1 µM, and 10 µM, respectively. Flux pack was hydrated overnight and calibrated for 30 min prior oxygen consumption rate (OCR) analysis.

The cellular bioenergetic parameters determined were ATP linked respiration, proton leak, maximal OCR, and reserve capacity. ATP linked respiration was derived from the difference between OCR at baseline and respiration following oligomycin addition. The change in OCR between antimycin A and oligomycin represented the amount of oxygen consumed due to proton leak. Maximal OCR was determined by subtracting the OCR after antimycin A addition from the OCR induced by FCCP. Lastly, the reserve capacity was calculated by the difference between maximal (FCCP) and basal respiration.

The intermediate turnover state, known as State 3.5 (the state app) and respiratory flux control were derived as detailed in [34]. In these experiments, the assumption is made that State 3 respiration is equivalent to the rate measured after addition of FCCP (State 3_{FCCP} or State 3u) and State 4 is the rate measured after addition of oligomycin (State $4_{oligomycin}$ or State 4o). These assumptions allowed the calculation of the apparent respiratory state of the cells using the equation

$$\text{State}_{\text{apparent}} = 4 - [(\text{Basal} - \text{Oligo})/(\text{FCCP} - \text{Oligo})]$$

where Basal represents the basal OCR, Oligo represents the oligomycin-insensitive OCR (proton leak), and FCCP represents the FCCP-stimulated OCR (maximal OCR). Using the same assumptions regarding the relative State 3 and State 4 respiration, the respiratory control ratio (RCR) was calculated as the State 3u rate divided by State 4o rate (maximal OCR/oligomycin insensitive OCR). Coupling efficiency is the proportion of the oxygen consumed to drive ATP synthesis compared with that driving proton leak and was calculated as the fraction of basal mitochondrial OCR used for ATP synthesis (ATP-linked OCR/basal OCR) [35]. Finally, the fraction of respiration that was used under routine conditions to produce ATP (phosphorylating respiration) was estimated as the ratio between ATP-linked OCR and maximal OCR (FCCP) (ATP linked OCR/maximal OCR) as described in [36].

2.7. Measurement of Reactive Oxygen Species Levels

Six-well plates were seeded with 150,000 cells per well and incubated with glucose (25 mM) for 24 h, followed by incubation with moringa extract (500 µg/mL) for 2 h at 37 °C with 5% CO_2. After incubation, cells were washed with PBS, detached with trypsin and resuspended in a final volume of 0.5 mL. Cell suspension was incubated with 1 µL of CellROX orange reagent for 30 min at 37 °C. The cell suspension was centrifuged at 600× *g* for 10 min and the pellet resuspended in 100 µL of PBS. Measures of 25 µL of the final suspension were used to quantify the fluorescence in a Tali image-based cytometer.

2.8. SDS-PAGE and Western Blot Analysis

Cellular pellet was resuspended in 0.1 mL of lysis buffer containing 20 mM Tris (tris(hydroxymethyl)aminomethane), pH 7.5, 150 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1% Triton X-100 and protease inhibitor cocktail. Cells were rapidly frozen

and thawed three times with liquid nitrogen to ensure maximal cell lysis and centrifuged at 5000× *g* for 15 min, supernatants were recovered and used for further analysis. 50 µg of protein (determined by bicinchoninic acid (BCA) analysis) were loaded into a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and run at 120 V for 90 min. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane previously activated with methanol. Membranes were incubated overnight with the primary antibody (anti UCP2—ab67241, anti SIRT3—ab189860, anti-acetyl lysine—ab80178, and anti OXPHOS—ab110413) at dilutions of 1:500, followed by 2 h incubation with the secondary antibody at dilutions of 1:1000. The intensity of bands was determined by Image Studio Lite v.5.2 software (LI-COR Biosciences, Lincoln, NE, USA).

2.9. BN-PAGE

Respiratory supercomplexes and complexes were solubilized using digitonin (a very-mild detergent) as described by [37] with minor modifications. Briefly, mitochondrial proteins isolated from HepG2 cells (10 mg/mL) were suspended in 3.5 mL of 50 mM Bis-Tris and 500 mM 6-aminocaproic acid, pH 7.0, and 140 µL digitonin (50% stock) were added to reach a detergent/protein ratio of 2:1 and incubated in this condition during 30 min. The mixture was centrifuged at 100,000× *g* for 30 min at 4 °C and supernatant was recovered.

Supercomplexes and complexes were loaded on a linear polyacrylamide gradient gel (4–10%) for Blue Native PAGE (BN-PAGE). Anode buffer contained 50 mM Bis-Tris/HCl, pH 7.0 and cathode buffer 50 mM tricine, 15 mM Bis-Tris, pH 7.0 and Coomassie Brilliant Blue R-125 dye (0.02%). The voltage was set to 35 V for 10 h at 4 °C and the run was stopped when the sharp line of the dye approached the gel front. Molecular weight of the respiratory complexes and supercomplexes was determined by their electrophoretic mobility and in-gel catalytic activity, using the digitonin-solubilized bovine heart mitochondria as standard. The intensity of bands was determined by Image Studio Lite v.5.2 software.

2.10. In-Gel CI and CIV Activities

The in-gel assays were performed as described by [38] using gel loaded with isolated digitonine-solubilized supercomplexes. CI activity was assayed in a buffer containing 5 mg MTT and 3.75 mg NADH in 10 mL of 10 mM Tris/HCl, pH 7.4. Once activity-staining appeared (10–20 min) reaction was stopped with fixing solution (50% methanol, 10% acetic acid). To assay the activity of complex IV the gel was incubated in 10 mL of 50 mM K_2HPO_4, pH 7.2, 10 mg of diaminobenzidine (DAB) and 2 mg of horse heart cytochrome c. After 30–40 min of incubation at 20–25 °C, the activity was observed as a brown precipitate and the reaction was stopped with the fixing solution. The intensity of bands was determined by Image Studio Lite v.5.2 software.

2.11. Statistical Analysis

Data were analyzed by one-way ANOVA using the SigmaPlot v.12.3 (Systat Software, Inc., San Jose, CA, USA). Differences among groups were considered significant when $p \leq 0.05$.

3. Results

3.1. Toxicity of the Extract and High Glucose

Previous studies have also shown that HepG2 cells are a better model than fresh human hepatocytes to define mitotoxicity [39] and it was reported that high glucose treatment at 25 mM for 72 h increased apoptosis in HepG2 cells through increase oxidative stress [40]. Recent reports demonstrated that the nano-micelle of *M. oleifera* seed oil remarkably induces mitochondrial apoptosis mediating cell death [41]. Moreover, *M. oleifera* aqueous leaf extract treatment resulted in a significant decrease in mitochondrial membrane potential (1 h) and ATP levels (3 h), followed by an increase in (6 h) ROS, caspase activation, proapoptotic proteins expression, and poly [ADP-ribose] polymerase 1 cleavage on different types of cells, including HepG2 [42]. Taken together, both high glucose and

M. oleifera extract could decrease cell viability by promoting mitochondrial dysfunction and oxidative stress. Therefore, we further investigated whether such experimental conditions affect our cell line.

In order to demonstrate that *M. oleifera* components and glucose do not have adverse effects on the overall viability of HepG2 cells, we performed an assay with different concentrations of the extract with or without high glucose. We did not find evidence of apoptosis in glucose treated cells. First, our MTT assay results showed that neither the extract nor glucose have negative effects over the viability of cells. Second, even at larger concentrations (500 μg/mL) *M. oleifera* extract did not showed any difference with respect to control group, in fact, the groups treated with glucose and the extract showed slightly higher viability than control cells (Figure 1). Hence, it is possible that *M. oleifera* extract can induce mitochondrial metabolic changes without affecting cell viability in early stages of treatment, supporting our previous results with an animal model. Although glucose had no negative effects on cells' viability, it influenced the mitochondrial respiration.

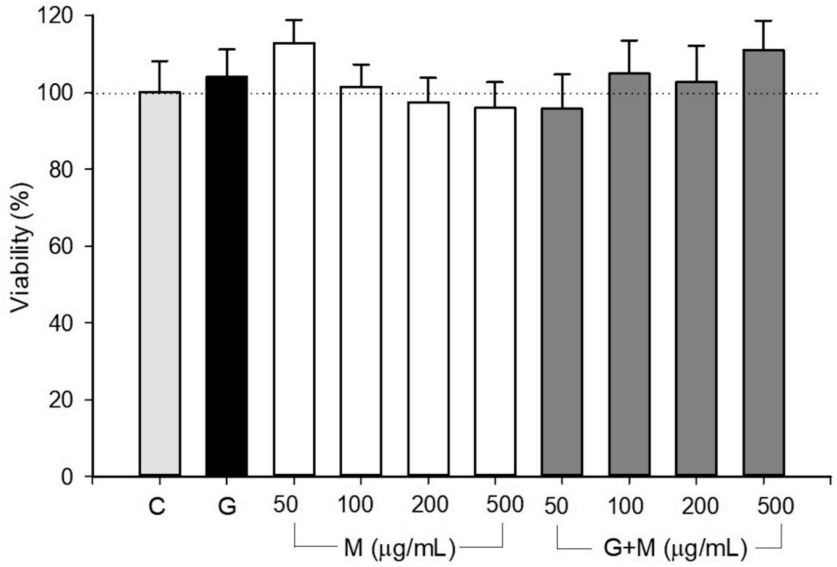

Figure 1. Viability of HepG2 cells treated with glucose and *M. oleifera* extract. Cells were incubated as described in material and methods section and incubated with 25 mM glucose (G) and different concentrations of *M. oleifera* extract (GM; 50–500 μg/mL). Data are expressed as the average ± SD from $n = 4$.

3.2. Effect of Moringa Extract on Mitochondrial OCR

To assess the effect of high glucose and *M. oleifera* extract on oxygen consumption rate (OCR) we used the Seahorse XF-24 analyzer. All OCR readings in the three groups were normalized to total protein concentration and the parameters analyzed were; the basal respiration (State II) is controlled by proton leak and substrate oxidation. In the presence of oligomycin, respiration is highly dependent on proton leak. The injection of uncoupling agent FCCP reestablishes electron flux and gives rise to maximum capacity of ATP generation or oxygen consumption. Finally, the residual oxygen consumption can be measured by injection of respiratory inhibitors rotenone/antimycin A.

Initially, the basal respiration did not differ between control and other treatments. HepG2 cells treated with high glucose showed no difference in basal respiration but the results demonstrated that the ATP-linked respiration and respiratory capacity was significantly reduced in the high glucose treated cells in contrast to untreated cells. Oligomycin was added at 7 min to inhibit ATP synthesis, interestingly, we observed that the lack of sensitivity of glucose-treated cells (HG cells) to oligomycin is likely because under these conditions the cells can compensate for mitochondrial impairment by utilizing glycolysis for ATP generation. In contrast, cells grown in high glucose and incubated with *M. oleifera* extract (GM cells) rely mostly on OXPHOS to produce ATP because they are more

sensitive to oligomycin. *M. oleifera* treatment not only increased ATP-linked respiration in HG cells but also completely restored their capacity compared to non-treated control cells (Figure 2). The proton ionophore FCCP was added at 13 min to assess the maximum possible oxygen consumption, the FCCP stimulated OCR and demonstrated that uncoupling of OXPHOS provokes an increase in proton leak across the inner mitochondrial membrane and effectively depletes the mitochondrial membrane potential. Interestingly, the stimulation of respiration by FCCP after oligomycin was substantially lower in the presence of high glucose. Moreover, rotenone and antimycin A was added at 19 min to inhibit electron flow through Complex I and III, limiting mitochondrial function with a decrease in OCR. Respiratory control ratio (RCR) was similar between control and GM treatment cells. Control cells RCR was 4.37 compared to HG cells RCR of 15.3, and GM cells RCR of 5.2, these values indicates high-quality mitochondria and efficient oxidative phosphorylation coupling. However, coupling efficiency of control cells was 0.4, which was decreased to 0.1 by the high glucose and significantly suppressed by *M. oleifera* extract (0.31). In addition, phosphorylating respiration in HG cells was 0.065. This was significantly lower that the phosphorylating respiration exhibited by control cells, 0.23. As a comparison, coupling efficiency in control cells was higher than both HG cells and GM cells. Additionally, the state app was not altered between different treatments (Control, 3.56; HG, 3.36, and GM, 3.49), suggesting no alteration in respiratory fluxes which could alter the oxidative phosphorylation machinery.

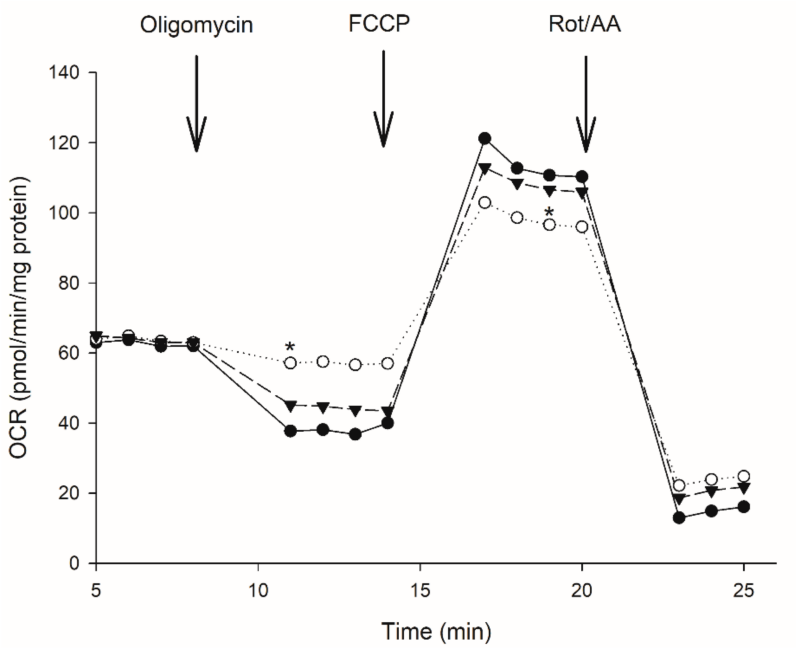

Figure 2. Absolute change in mitochondrial function in intact HepG2 cells treated with glucose 25 mM (white circle) and *M. oleifera* extract 500 µg/mL (inverted gray triangle). The addition of oligomycin at 5 min inhibits ATP production resulting in a decrease in oxygen consumption rate (OCR). The OCR increases in all treatments following the addition of FCCP at 13 min (uncoupled state). Electron transport chain inhibitors mix (Rotenone and Antimycin A) decrease oxygen consumption rates to very low levels inhibiting total mitochondrial respiration at 18 min. * significant difference against control $p \leq 0.05$.

3.3. Uncoupling Protein Level (UCP2)

To assess whether the above respiration profile data were directly linked to some defect inside the respiratory chain or induce of alternative components, we reasoned that UCP2 activation is likely, at least partially, responsible for the reactive oxygen species inhibitory effect of high glucose in HepG2 cells. In addition, the presence of high glucose caused an increase close to 50% in proton

leak (uncoupling protein activity). In support of this hypothesis, we observed that the combined treatment of high glucose and *M. oleifera* extract enhances ATP production and maximal respiration (Figure 2). Our hyperglycemic model showed more than three-fold increase in UCP2 levels in those cells exposed to 24 h of high glucose (Figure 3). We can assume that UCP2 is highly upregulated during hyperglycemia in liver and its presence correlates with the adaptability of hepatic cells to high concentrations of extracellular glucose. Interestingly, after only 2 h of incubation with *M. oleifera* extract, UCP2 levels normalized (Figure 3). These results suggest that antioxidant properties of extract regulate the UCP2 expression in HepG2 and possibly the ROS production.

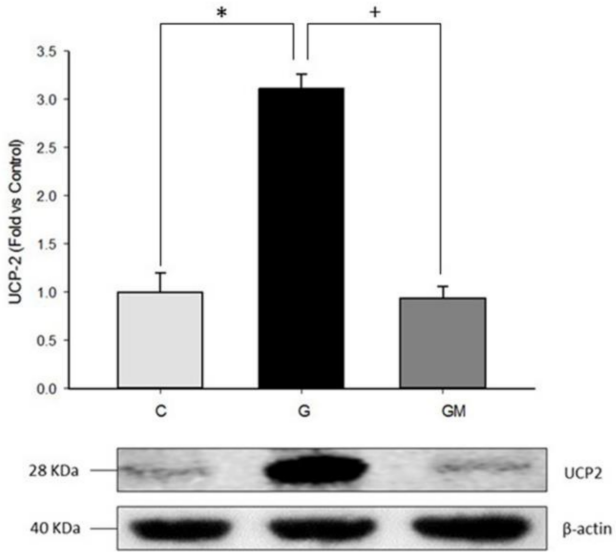

Figure 3. UCP2 protein levels in HepG2 cells treated with glucose 25 mM (G) and *M. oleifera* extract 500 µg/mL (GM). Cells without treatment represent control condition (C). * significant difference against control $p \leq 0.05$. $^{+}$ significant difference against G cells $p \leq 0.05$. Data normalized to control group using β-actin.

3.4. Reactive Oxygen Species Levels

Higher production of ROS is often stated as both cause and consequence of mitochondrial dysfunction [43]. There is evidence that supports the fact that under hyperglycemic conditions ROS production is significantly higher [44]. To identify the role of ROS in the context of hyperglycemia and mitochondrial dysfunction, we carried out fluorescence tests to HG cells and GM cells. However, after performing CellROX orange fluorescence test, data from the Tali cytometer showed no significant changes of ROS between control cells (5 ± 1.39% of cells) and those treated with glucose (4 ± 1.42%), nor the combination glucose plus *M. oleifera* (4 ± 1.42%). Therefore, we performed repeated tests using Amplex Red ROS-specific probe, and all data were negative for high glucose induced ROS production (data not shown). Hence, the lack of ROS overproduction in our model suggests that diverse metabolic adjustments have occurred at the level of the OXPHOS proteins, which allows cells to adapt to the high glucose environment, and that some regulatory mechanisms could be playing an important role.

3.5. OXPHOS Activities

In order to evaluate this possibility, we measured both the specific activities and analyzed the expression level of individual respiratory complexes. Initially, we evaluated the activity of Complex I and IV to establish if respiration disruptions observed during hyperglycemia are consequence of protein activities. Interestingly, Complex I activity was significantly lower in those HG cells (Figure 4b), correlating with the disruptions observed in respiratory rates in the same samples (Figure 2). As for Complex IV, we found no significant differences among groups (Figure 4c). Despite the clear effect

of high glucose over Complex IV protein levels, this protein can modulate its activity to adapt and sustain cell viability. We were not able to measure Complex III activity, however, due to the lower levels of Complex III and the diminished activity of Complex I found in HG cells, we can assume that these two complexes are responsible for the respiratory alterations observed in our hyperglycemic model. As shown in Figure 4a, Complex III significantly decreases when the cells are treated with high glucose, interestingly this effect is reverted after only 2 h of incubation with 500 µg/mL of *M. oleifera* extract. On the other hand, Complex IV (Figure 4a) is also decreased in those cells incubated with high glucose, but moringa extract can revert this negative effect. Although protein levels are not as high as control group, these are still significantly different from those found in HG cells. This finding is consistent with [45], who used mass spectrometry analysis to report a 46% decrease in Complex III levels alongside with 20–30% downregulation of subunits from Complex I–IV in cardiac and skeletal muscle mitochondria from diabetic rats. In other reports, proteomic analysis of obese and type 2 diabetic skeletal muscle mitochondria, found less presence of several mitochondrial proteins, including those in the electron transport chain in both human [46] and mice samples [47]. This suggests that tissues with high energy demand or involved in glucose homeostasis are the first ones to suffer from alterations in OXPHOS subunits under hyperglycemic conditions.

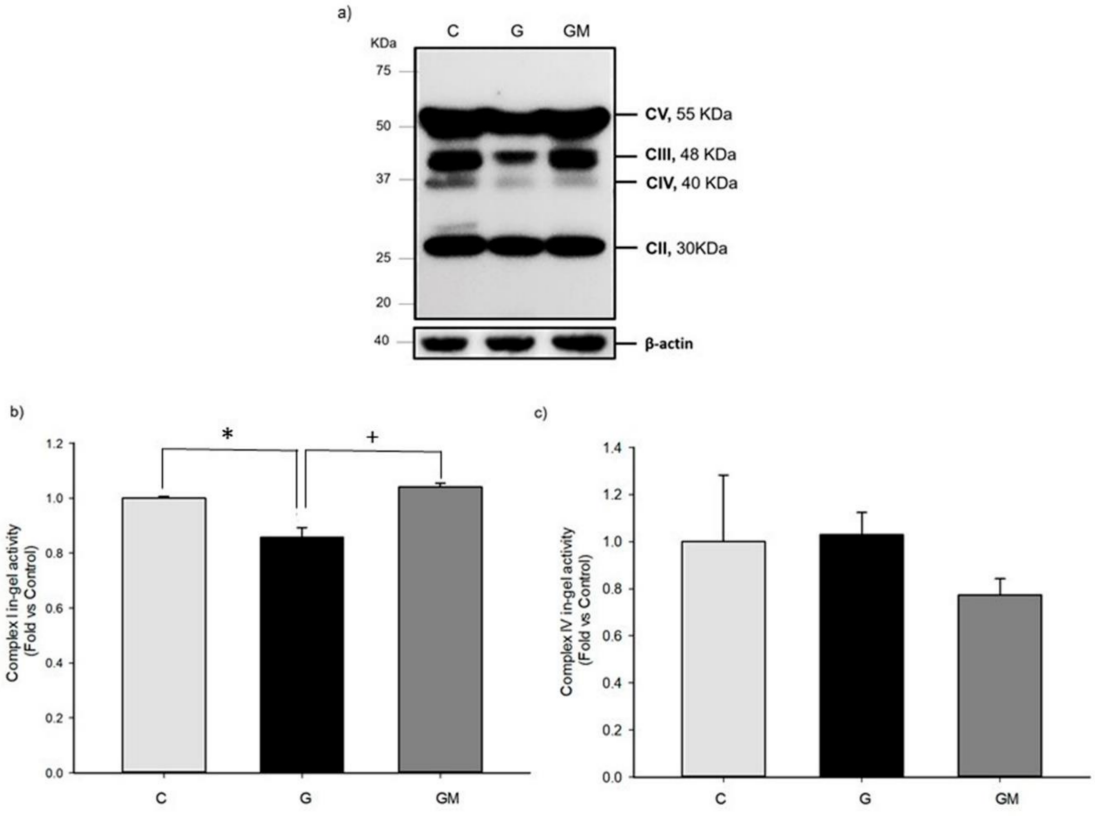

Figure 4. (**a**) Characterization of OXPHOS proteins expressed in HepG2 cells during high glucose with or without *M. oleifera* extract. OXPHOS cocktail specificity demonstrated by Western blot of MTC01 subunit of CIV, SDHB subunit of CII, UQCRC2 subunit of CIII, NDUFB8 subunit of CI, and ATP5A subunit of CV. BN-PAGE. Activity of CI (**b**) and CIV (**c**) in isolated mitochondria from HepG2 cells under normal (C), glucose 25 mM (G), and 500 µg/mL *M. oleifera* extract (GM) conditions. * significant difference against control $p \leq 0.05$. $^{+}$ significant difference against G cells $p \leq 0.05$. Data normalized to control group using β-actin.

3.6. Mitochondrial Supercomplex Levels

Recent experimental evidence has replaced the random diffusion model of electron transfer with a model of supramolecular organization based upon specific interaction between individual respiratory complexes [48]. Supercomplexes (SC) is the term given to associations between different mitochondrial complexes [49]. Three of the electron-transfer complexes form SC and several functions have been attributed to SC in mitochondria, and although information regarding this matter is controversial, there are reports of SC modulating ROS formation and facilitate efficient energy generation [50,51].

Since we observed changes in protein levels from Complexes III and IV, which are important components of SC, we conducted experiments using BN-PAGE to assess the amount of SC in our model to establish if complex interaction is lost under hyperglycemic conditions. Same as in individual complexes, exposition to high glucose lowers the overall interaction between mitochondrial complexes. Again, the presence of *M. oleifera* extract reverted this effect (Figure 5) indicating that components in the extract are not only capable of preserving OXPHOS protein levels, but also preserving the interactions between them, ensuring a better mitochondrial metabolism.

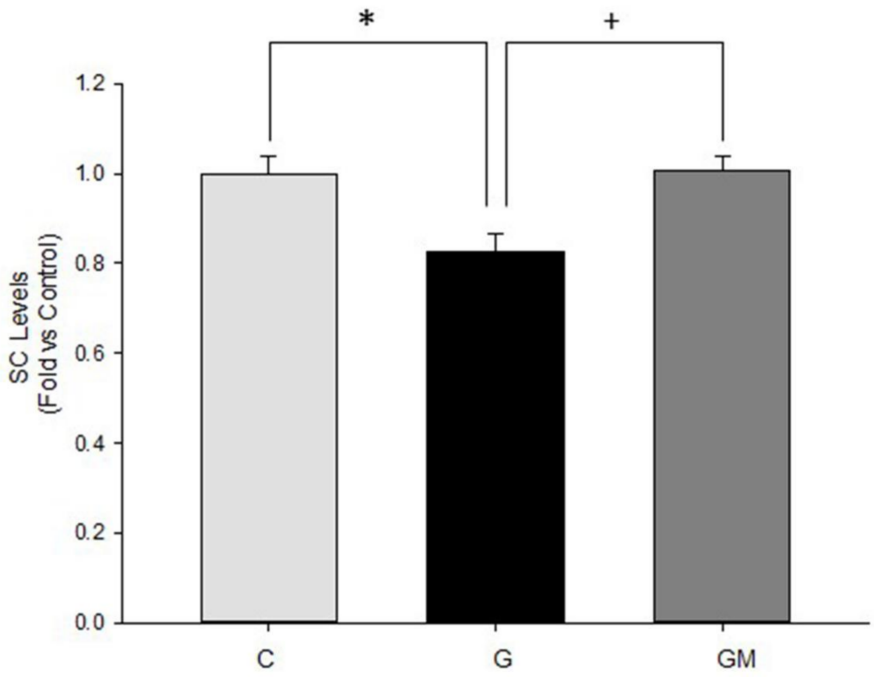

Figure 5. Interactions between mitochondrial complexes in HepG2 cells under normal (C), glucose 25 mM (G), and 500 µg/mL *M. oleifera* extract (GM) conditions. * significant difference against control $p \leq 0.05$. $^{+}$ significant difference against G cells $p \leq 0.05$.

3.7. Acetylation Levels by SIRT3

Protein post-translational modification is an important process for quickly and transiently modifying the structure of a protein by the covalent addition of functional groups, proteolytic cleavage of regulatory subunits, or degradation of entire proteins, which causes changes in enzyme activity as well as interfering or aiding protein-protein interactions. To date, no work has specifically analyzed the role of post-translational modification in SC assembly or function, but there is a large body of information on post-translational modification of individual respiratory complexes and other mitochondrial proteins [52].

Our results indicated that acetylation percentage is higher in those cells treated with high glucose, and after 2 h of exposure to *M. oleifera* extract, acetylation levels diminished significantly, even below control levels (Figure 6a). Our results regarding SIRT3, showed that there is no significant difference

among groups, however, levels in *M. oleifera* treated cells are slightly higher than those found in high glucose treated cells (Figure 6a), existing the possibility that lower acetylation levels in cells treated with the extract are due to higher amounts of SIRT3, and of course, other SIRTs. Our data is contrasting with previous findings where the content of SIRT3 was heavily decreased in diabetic pancreas and lung [53], possibly the alterations in SIRT3 in diabetes are likely tissue dependent [54]. Moreover, when total mitochondrial protein acetylation profile was assessed by anti-acetylation western blot analysis, an increased acetylation on numerous proteins could be detected in diabetes [55], which corresponds with our results (Figure 6b).

Although we were not able to identify the acetylated mitochondrial proteins, our results clearly show that the level of acetylation in the high glucose treated cells was increased by 40% with respect to the control group. In addition, the acetylation levels were decreased three times with respect to the group treated with high glucose plus *M. oleifera* (Figure 6b). It is difficult to determine which biochemical feature is affected by acetylation; enzyme activity, protein-protein interactions, protein–DNA interactions, stability, localization, allostery, and others.

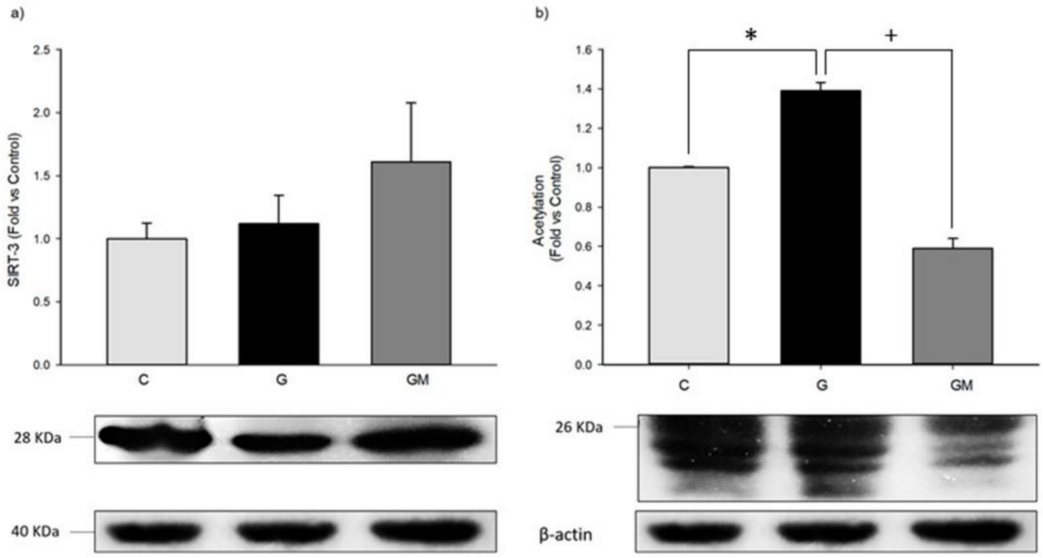

Figure 6. Sirt3 (**a**) and overall acetylation (**b**) protein levels in HepG2 cells under normal (C), 25 mM glucose (G) and 500 µg/mL *M. oleifera* extract (GM) conditions. * significant difference against control $p \leq 0.05$. $^{+}$ significant difference against G cells $p \leq 0.05$. Data normalized to control group using β-actin.

4. Discussion

There is significant evidence that energy production is impaired during diabetes; however, the molecular events involved are poorly understood. Many compounds isolated from *M. oleifera* have been reported to show antidiabetic and biological properties [56,57]. However, the molecular targets in which the phytochemicals of the *M. oleifera* extract act are unknown. In addition, a recent interest has been devoted to studying the effects on mitochondria of natural compounds as quercetin, resveratrol, and curcumin [58]. Many of these compounds turned out to exert their functions by affecting mitochondrial function, either directly, by inhibiting specific enzymes, or indirectly, by modulating signal from or to mitochondria [59–61].

Reports in C57BL/6 mice, showed that quercetin clearly reduced the expression levels of mitochondrial proteins that control mitochondrial dynamics. Interestingly, they found that quercetin reduced the activity only in monomeric Complex IV [62]. Our previous findings with liver isolated mitochondria of STZ-treated rats demonstrated changes in both amount and composition of SC [9],

which were replicated in HepG2 cells. Our results show that high glucose also reduced the levels of OXPHOS proteins subunits from respiratory complexes (Figure 4a). Specifically, UQCRC2 subunit of Complex III and MTC01 subunit of Complex IV were significantly reduced. While SDHB subunit of Complex II, and ATP5A subunit of Complex V were not decreased in high glucose treatment. Interestingly, we were not able to detect the representative band of the NDUFB8 subunit of Complex I in either control cells or those treated with high glucose and/or *M. oleifera* extract. However, all groups present significant specific enzymatic activity of this complex. One possible explanation is that the antibody recognition region into the Complex I protein subunit is not available because this complex is always associated with other components of the respiratory chain. In addition, the inhibition of Complex I in high glucose treated cells would cause a decrease in energy supply that would in turn lead to a higher AMP/ATP ratio, and the concomitant activation of AMP-activated protein kinase, although an increase in UCP2 levels could also produce the same result [63]. These results support the idea of several groups who have proposed a stabilizing factor for respiratory supercomplex assembly, cytochrome c oxidase (COX) subunit 7a-related polypeptide (COX7RP) [64,65]. Recently, it has been shown that a metabolic phenotype of Cox7rp knockout mice exhibit lower blood glucose levels after insulin or pyruvate injection. Notably, ATP synthesis rate was reduced in Cox7rp knockout mice liver, in accordance with decreased percentages of Complex III subunit RISP and Complex COX1 involved in respirasome fraction [65]. This result suggests that COX7RP-mediated mitochondrial respiration plays crucial roles in the regulation of glucose homeostasis and its impairment will lead to the pathophysiology of metabolic states.

On the other hand, computational studies have shown that up to 20% of mitochondrial proteins can be acetylated on their lysine residues and are putatively regulated by SIRTs [43]. Three NAD-dependent deacetylases; SIRT3–5; are localized to the mammalian mitochondria. SIRT3 has been implicated in regulating metabolism by deacetylating Complex I subunit NDUFA9 and affect NADH-dependent respiration in mice, as well as α and OSCP subunits from F0F1ATPase and the SdhA subunit from Complex II, demonstrating the role of acetylation/deacetylation in the regulation of oxidative phosphorylation [17,18]. However, from the 700 acetylated mitochondrial proteins, only 26 proteins that display functional effects when acetylated [66]. Several acetylome proteomic studies have identified many lysine-acetylated mitochondrial proteins, including six tricarboxylic acid (TCA) cycle proteins, 26 proteins involved in oxidative phosphorylation, 27 β-oxidation, 8 associated with amino acid metabolism, 10 with carbohydrate metabolism, 3 with nucleotide metabolism, and 2 with the urea cycle [67,68]. With respect to mitochondrial dehydrogenases, 21 were lysine acetylated, among them, 9 subunits of NADH dehydrogenase (Complex I). In this study, Figure 6b shows also that mean value of acetylation of high-glucose treated cells was significantly (at $p < 0.05$) increased compared with control cells. Treating these high-glucose treated cells with *M. oleifera* extract significant (at $p < 0.05$) ameliorated with that high-glucose treated cells. In contrast, Figure 6a shows also that the mean value of SIRT3 was not significantly increased due to high glucose treated cells. Interestingly, treating these cells with *M. oleifera* extract no significantly reduced the SIRT3. These data suggest that other members of SIRTs family may be participate into mitochondria as SIRT1 or 5. However, it is necessary more experimental evidence.

Increased oxidative stress has been hypothesized to activate uncoupling protein 2 (UCP2) which is a regulator of ROS production in the inner membrane of mitochondria. In addition, the liver is the largest metabolic organ in the human body, and mitochondrial proton leak accounts for 20–30% of the oxygen consumption of isolated resting hepatocytes [69]. In the liver, UCP2 has been localized to Kupffer cells, with very low or undetectable levels in hepatocytes. However, this expression pattern appears to be the opposite in fatty liver [70]. Accordingly, we found that the levels of UCP2 was increased in HG cells (Figure 3) and *M. oleifera* extract caused remarkable decrease in UCP2 expression, this last finding is probably due to a direct modulation of mitochondrial environment by components in the extract, where UCP2 is no longer needed in higher levels. This is supported by the fact that *M. oleifera* extract is also capable of restoring alterations in mitochondrial free fatty

acids accumulation, a known activator of UCP2 [71–74], and previous reports where UCP2 levels are regulated by quercetin [74], a component present in our extract [33]. These results suggest that HepG2 protection against high glucose injury is associated with the upregulation of UCP2. Thus, our results are in accordance with findings of non-alcoholic fatty liver disease (NAFLD) [75].

NAFLD is part of the metabolic syndrome with insulin resistance as a primary underlying derangement. Available evidence suggests that UCP2 may theoretically contribute to pathogenesis of NAFLD [76]. In addition, the expression of SIRT1 is significantly lowered and UCP2 increased in the liver of rats with diabetes and NAFLD. It was proposed that UCP2 regulates the activity of SIRT3 through sensing the energy levels and, in turn, maintaining the mitochondrial steady state, which demonstrates a cytoprotective effect on cerebral ischemia-reperfusion injury [77]. UCP2 induces mitochondrial proton leak and increases susceptibility of non-alcoholic steatohepatitis liver (NASH) to ischemia-reperfusion injury [78]. Moreover, cardiolipin a phospholipid located at inner mitochondrial membrane, plays an important role in several processes involved in mitochondrial bioenergetics and apoptosis. Cardiolipin peroxidation has been associated with the destabilization of mitochondrial respiratory supercomplexes could be another factor contributing to ROS generation and to mitochondrial bioenergetic decay in NAFLD [79].

On the other hand, metformin can increase the expression of SIRT1 and reduce the expression of UCP2, with negative correlation between the expression of SIRT1 and UCP2 [80]. Berberine can downregulate the expression level of UCP2 mRNA and UCP2 proteins of hepatic tissue from NAFLD rats [81]. Interestingly, dietary polyphenols have been identified to offer a potential therapy for NAFLD and its progression to nonalcoholic steatohepatitis [82]. Several polyphenols, such as kaempferol, have been reported to activate both SIRT1 and SIRT3 [83]. Polyphenols found in beverages, such as red wine and grape juice, could bear an effect on energy metabolism, being able to increase UCP2 expression, by increasing energy expenditure, even when administered in a high fat diet situation [84]. Thus, in line with our results, decreased expression of the mitochondrial biogenesis transcription factors PGC1α and NRF1, and decreased expression of respiratory Complex I and V subunits NDUFS8 and ATP5G1 in the HepG2 cell model of steatosis have been reported. The treatment with different polyphenols protected by more than 50% against the oleic acid induced increase in ROS and prevented the decrease of UCP2 [85].

Taken together, we proposed a model to explain our data. Under physiological conditions (Figure 7a), mitochondria play a key role in energy metabolism by generating most of the energy used by mammalian cells. The redox power from organic acids oxidation is provided to the respiratory chain by reduced donors (NADH + H^+; $FADH_2$) or directly by specific dehydrogenases via electron-transfer complexes to the quinone pool before reducing final electron acceptor (molecular oxygen). Electron flow is conveyed along the mitochondrial respiratory chain and part of its energy is converted to an electrochemical force by pumping out protons across the inner mitochondrial membrane. This generates electrochemical gradient (also called the proton motive force, pmf) that can be used to synthesize ATP or exchange proteins or ions (Ca^{2+}) across the inner mitochondrial membrane. The efficiency with which reduced equivalents are used to generate ATP by oxidative phosphorylation is dependent on mitochondrial coupling. Uncoupling the proton transport across the membrane participates in the regulation of energy homeostasis and defaults in electron transfer can enhance ROS production. Under conditions of excess in energy intake (high glucose) and tight mitochondrial coupling (Figure 7b), pmf can rise to a maximum. Thus, mitochondrial respiratory complexes are highly reduced and may release electrons directly to oxygen resulting in a higher ROS production that could alter oxidative phosphorylation system and lead to a drop of mitochondrial ATP levels. Thus, excessive ROS production would lead to induced uncoupling protein 2 (UCP2). UCP2 is thought to protect against oxidative stress although, alternatively, it could play an energy dissipation role. Consequently, in our study, the increase of UCP2 associated with the increase of proton leak (uncoupling state) may improve the mitochondrial NAD^+/NADH ratio by suppressing the ATP synthesis and ROS production. SIRT3 is a key regulatory protein, which can sense the NAD^+ levels. Therefore, it

might be possible that UCP2 increases the NAD^+/NADH ratio to activate SIRTs in high glucose conditions. Furthermore, treatment of high glucose treated cells with *M. oleifera* extract (Figure 7c), increases the levels of acetylation and lowers the UCP2 protein. *M. oleifera* also increases the levels of supercomplexes to optimize the Complex I activity and coupling state. In accordance with these results, we also observed a recovery of the disturbed bioenergetics homeostasis (RCR, coupling efficiency and phosphorylating respiration). Finally, when energy stores are plentiful, Krebs cycle intermediates accumulate, and citrate is transported back into cytoplasm where is converted to acetyl-CoA, which is the first step of endogenous fatty acid synthesis and lipogenesis is a central abnormality in NAFLD (Figure 7 and [86]. However, this last metabolic alteration requires further investigation.

In conclusion, *M. oleifera* treatment significantly reduced acetylation of mitochondrial proteins and subsequent increase in amount of supercomplexes and Complex I activity, while it dramatically decreased UCP2 expression in high glucose treated HepG2 cells. *M. oleifera* could be a potential source of mitochondrial drugs for diabetes and NAFLD.

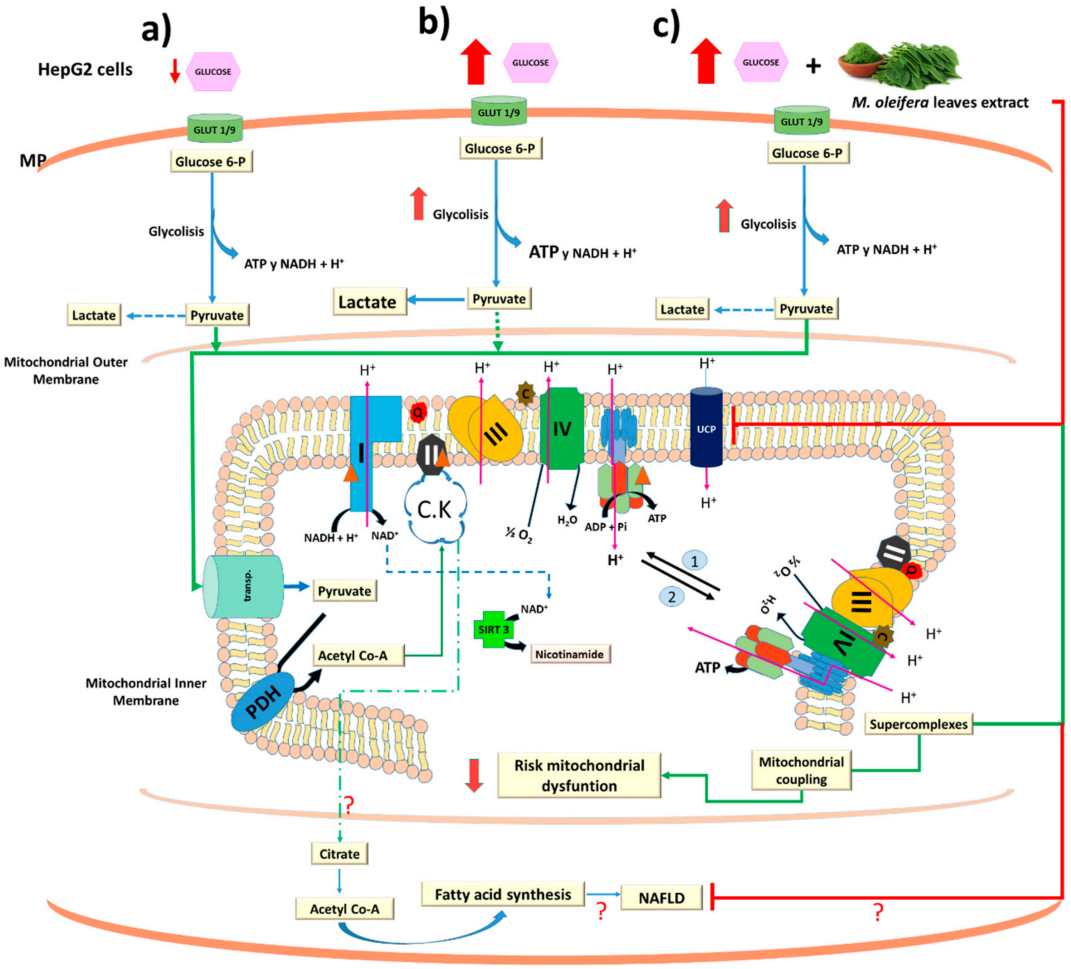

Figure 7. Proposed model for uncoupling protein 2-acetylation by sirtuins signaling pathway on HepG2 treated with high glucose and *M. oleifera* extract. The solid lines indicate carbon skeletal main flux to mitochondria (**a**). While that punted lines indicates the secondary pathways. PDH, pyruvate dehydrogenase; CK, Krebs cycle. The numbers 1 and 2 indicates the dissociation and association of the mitochondrial supercomplexes. Red and green lines indicate inhibition and activation of metabolic process by high glucose (**b**) and *M. oleifera* extract (**c**), respectively. Glut 1 and Glut 9 as major contributors to glucose influx in HepG2 cells [87]. MP, plasma membrane. Red triangle indicates potential site of acetylation in mitochondrial complexes.

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RESEARCH ARTICLE

Lactate Dehydrogenase in Hypertensive Disorders in Pregnancy: Severity or Diagnosis Marker?

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Abstract

Background: Lactate dehydrogenase has had an exciting journey as a utility marker in different illnesses, but currently, its clinical utility has been relegated to confirm hemolysis, as a tumor marker, and as a diagnostic biomarker of preeclampsia. The findings of lactate dehydrogenase concentrations taking reference values to healthy persons are not consistent when these are related to hypertensive disorders in pregnancy, mainly to begin symptoms or little severity presentation. The goal in this work was to evaluate the maternal serum concentration of lactate dehydrogenase and its utility as a severity or diagnosis marker for hypertensive disorders in pregnancy.

Methods: In this retrospective study, we included 5,558 cases of HDP and 800 healthy pregnancies. HDP classification and LD values were collected from the medical records in the paper chart.

Results: The prevalence of HDP in our hospital was approximately 6.4 ± 0.1%. We found a tendency toward increases in median LD concentrations with the increasing severity of HDP and found a positive correlation ($p = 0.037$) or error probability of 0.037% between LD concentrations and severity of HDP in Mexican pregnant women.

Conclusion: Serum LD concentration in HDP is a marker of severity, diagnosis and adverse maternal outcomes.

Keywords

Lactate dehydrogenase, Hypertensive disorders in pregnancy, Diagnostic markers

Introduction

Lactate dehydrogenase (LD) is a cytoplasmic enzyme that is widely expressed in tissues and cells. LD is an enzyme in the glycolytic pathway catalyzes the oxidation of L-lactate to pyruvate with the mediation of nicotinamide adenine dinucleotide (NAD+) as the hydrogen acceptor. This reaction is reversible and can be detected in the laboratory in serum samples by measuring LD activity in terms of the rate of dihydronicotinamide adenine dinucleotide dehydrogenase (NADH) production

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determined spectrophotometrically at 340 nm [1,2]. LD is a critical serologic marker for diagnosis, staging/prognosis, and recurrence, and monitoring of germ cell tumors [3], as well as for multiple myeloma, another malignant disease wherein high LD levels are associated with disease severity and poor prognosis [4,5]. Serum LD levels increase in proportion to the clinical severity of idiopathic pulmonary arterial hypertension and have a strong, independent association with the long-term mortality of these patients. Assessing the potential role of LD as a biomarker and mediator involved in the pathogenesis of idiopathic arterial hypertension might be worthwhile [6]. LD has had an exciting journey as a utility marker in different illnesses, but currently, its clinical utility has been relegated to confirm hemolysis, as a tumor marker, and as a diagnostic biomarker of preeclampsia (PE) [3,7]. However, the findings of LD concentrations taking reference values to healthy persons are not consistent when these are related to hypertensive disorders in pregnancy (HDP), mainly to begin symptoms or mild PE.

The HDP are among the leading causes of maternal and perinatal morbidity and mortality worldwide [8]. The public classification system was adopted by the National High Blood Pressure Education Program (NHBPEP) Working Group in 1990 and subsequently endorsed by 46 medical organizations. The updated version in 2000 has become a standard that the American College of Obstetrics and Gynecology (ACOG) follows. From the NHBPEP original reports, guidelines from international societies have emerged, each one with their evidence, although many with similar recommendations [9]. The HDP should be classified as pre-existing hypertension, gestational hypertension, preeclampsia, or others hypertensive effects based on different diagnostic and therapeutic considerations. Hypertension in pregnancy is defined by systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg [10].

Dramatic changes in the cardiovascular system occur throughout gestation beginning soon after conception, presumably with the objective of increasing blood flow and nutrient delivery to the fetal-placental unit. The Healthy pregnancy is associated with increased endothelium-mediated relaxation, blunted response to vasoconstrictors, and increased flow-mediated dilation [11]. Modification of the placental bed arteries to reach a high-flow, low-resistance status to support this increased blood flow is achieved by extravillous trophoblast-mediated remodeling of spiral arteries, with a replacement of the endothelium by trophoblasts [12]. The link between abnormalities in trophoblast invasion and generalized maternal endothelial dysfunction seen in HDP, particularly in preeclampsia, maybe via release of placental factors, such as syncytial knots, shedding of syncytiotrophoblast basement membrane fragments (STBM), leukocyte and platelet membrane particles, activated neutrophils, cytokines, growth factors, angiogenic factors, and hormones [13]. These factors will interact with the maternal vascular endothelium, which may already be damaged and can cause maternal endothelial cell damages. The STBM may also damage the endothelium and activate neutrophils, and this may lead to endothelial dysfunction as part of the widespread intravascular inflammation [14]. Evidence for endothelial dysfunction in preeclampsia includes reduced *in-vitro* endothelium-dependent dilatation of isolated vessels, increased vascular reactivity in response to vasoconstrictor stimuli, and elevated biomarker levels associated with endothelial activation and injury [12]. Detection of high-risk patients with increased LD levels, as a marker of endothelial damage by HDP, mandates close monitoring and correct management to decrease both maternal and fetal morbidities [15]. In the present study, we evaluated the maternal serum concentration of LD and its utility as severity or diagnosis marker for HDP.

Material and Methods

In this retrospective study of 10 years, we included all women diagnosed with any HDP who were admitted to the Gynecology and Obstetrics Department of the Hospital General de Durango, Mexico, between January 2008 and December 2017.

Pregnant women with any HDP were identified by final diagnosis of the patient at discharge in the hospital and recruited by the archive and statistic department in agreement of the ICD-10 code. Patients with HDP diagnosed from localities outside the Durango state and women with HDP associated with trophoblastic disease were excluded. HDP diagnosis was confirmed by medical record review by the principal investigators. Gestational hypertension was defined by systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg on at least two occasions 6 hours apart, without proteinuria. Preeclampsia was defined as > 20 weeks gestation with incident hypertension (defined as a systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure 90 mmHg on at least two occasions 6 hours apart) and proteinuria (300 mg protein excreted over 24 h, or 30 mg/dL in a random urine sample or 1+ protein on urine dipstick). Eclampsia was defined by seizure occurrence in women with preeclampsia that cannot be attributed to other causes. The hemolysis, elevated liver enzymes, and low platelet (HELLP) syndrome in preeclamptic or eclamptic women was defined by platelet count less than 100,000 cells/mm^3, liver enzymes more than twice the normal value, and the presence of microangiophatic haemolytic anemia, or observation of burr cell schistocytes and polychromasia on peripheral blood smear observation. Healthy pregnancies were defined as those normotense pregnant women without complications before, during and later pregnancy resolution.

Maternal demographic data including place of birth, age, gravity, gestational age at delivery, mean arterial pressure (MAP), HDP classification, and LD values were

collected from the medical records in the paper chart. LD values were taken from the first laboratory examination during admission. The LD concentrations were determined through a dry chemistry method in Johnson & Johnson Vitros® 5.1 FS analyzer by (Ortho Clinical Diagnostics 1001 U.S 202 Raritan, NJ 08869), validated with daily internal quality control and monthly by external quality assurance programs.

Statistical analysis

The SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis; the clinical characteristics of the HDP sub-types were expressed as mean ± standard deviations (SD) or median and interquartile range (IQR). The mean of the continuous values was compared using the Student's t-test after testing for normality using the Kolmogorov-Smirnov test. A p-value equal to less than 0.05 was considered statistically significant. Mann-Whitney U-test, or student's t-test depending on the normality distribution, was performed to compare the groups. To calculate bi-variate correlation between LD values and HDP severity expressed in the probability that LD concentrations are a severity marker of HDP, we calculated the Spearman range correlation, and data were represented in graphic distribution by error bars later. Finally, a baseline was obtained with IQR (Q1 - Q3) of LD values for each HDP classification, and reference values were established for each classification.

Results

Ethical approval was obtained from the Institutional Review Boards of the Hospital where this retrospective study was conducted. In our hospital 107,937 patients were attended in the gynecological & obstetrical service during 10 years, and 86,202 deliveries occurred during the same period, including fetal deaths 20 weeks gestation. A total of 5,552 women presented with pregnancy complicated with any HDP category in agreement with the criteria of NHBPEP and Technical Guidelines for Diagnosis, Prevention and Management of Preeclampsia-Eclampsia of Health Ministry in Mexico. The case distribution in 2008 to 2017 with the different sub-classifications of HDP, including 800 healthy pregnancies, is shown in Table 1. All cases were confirmed after the medical records were reviewed. The prevalence of HDP

Table 1: Lactic dehydrogenase values for different hypertensive disorders of pregnancy including normo-evolutive pregnancy.

Hypertensive disorders of pregnancy	Cases (n)	LD concentration (IU/L)	Standard deviation	Q1 Minimum value (IU/L)	Q3 Maximum Value (IU/L)
Normo-evolutive pregnancy	800	274.49§	101	201	360
Gestational hypertension	2,057	515.05¢	339	400	565
Mild preeclampsia	1,089	537.13§	122	463	567
Severe preeclampsia	1,817	654.92§	222	522	729
Eclampsia	172	747.56§	219	556	921
Severe preeclampsia + HELLP	361	1,492.4¢	1,178	790	2,066
Eclampsia + HELLP	56	4,634.79¢	3,855	2,117	4,898

§Media; ¢Median.

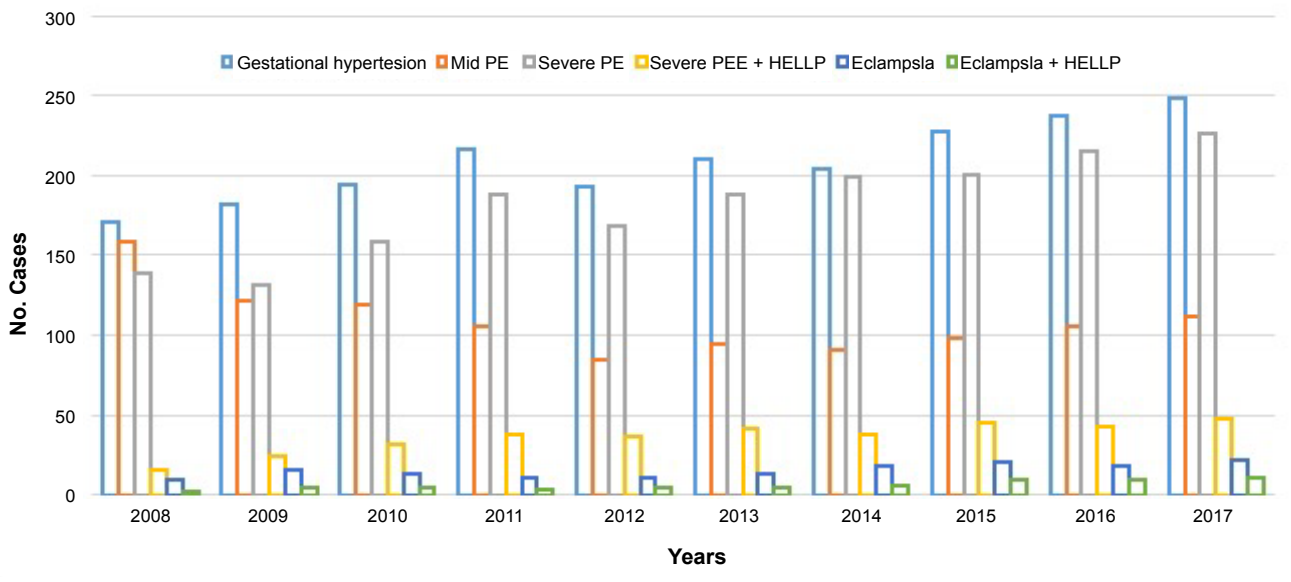

Figure 1: Cases of Hypertensive disorders of pregnancy from 2008 to 2017.

in our hospital was about 6.4 ± 0.1%. The occurrence of HDP has increased each year in proportion with the pregnant women attended. However, the proportion related to HDP severity had been changed with respect to mild PE and severe PE because severe PE had been increasing, whereas mild PE had been decreasing over these 10 years possibly by changes in clinical criterial for classification of mild and severe preeclampsia; however, gestational hypertension and eclampsia remained stable (Figure 1). With respect to the frequency of HDP: Gestational hypertension 37.1% (n = 2,057), mild PE 19.6% (n = 1,089), severe PE 32.7% (n = 1,817), eclampsia 3.1% (n = 172), severe PEE with HELLP 6.4% (n = 361), and eclampsia + HELLP only presented, 1.01% (n = 56), of all cases, respectively. The mean of the chronologic age of women was 24.8 ± 7.3 years; the mean for gestational age was 36.7 ± 4.0 weeks. The mean number of pregnancies was 2.29 ± 1.6, and MAP was 108.8 ± 18.3 mmHg. With respect to the chronological age of pregnant women, those who had eclampsia had the lowest mean age (21.6 years ± 6.2 SD), compared with media the total of the women.

To calculate the LD reference concentrations for HDP; we include 14.5% (n = 800) LD values of healthy pregnancies which are shown in Table 1. So, LD reference values were stablished for normo-evolutive pregnancies and pregnancies complicated with HDP. Then, we found a tendency to increase the mean of LD concentrations in relation to HDP severity and PE complicated with HELLP syndrome. After establishing the data of non-parametric distribution, and Spearman range correlation analysis, we found a correlation (p = 0.037) or error probability of 0.037% between LD concentrations and HDP severity in Mexican pregnant women (Figure 2). Likewise, the median and interquartile ranges (IQR) were established for LD values to find reference ranges through Q1 and Q3 values and, propose a new reference range for Mexican normo-evolutive pregnant women and Mexican pregnant women complicate with HDP (Table 1). Finally we established a baseline of LD values related to HDP (Figure 3).

Discussion

Based on the World Health Organization, HDP has been the second leading cause of maternal death globally to date [16], but it is the leading cause of maternal death in Latin America with up to 25% cases [17,18]. The most at risk age groups are young mothers aged between 10 and 24 years, and there are groups that present HDP with more severity [19]. Our population studied had a similar risk, with more prevalence of severe PE and eclampsia in young women (21.6 ± 6.2 years). In contrast, change in the decrease of frequency for mild PE and increase for severe PE was found in

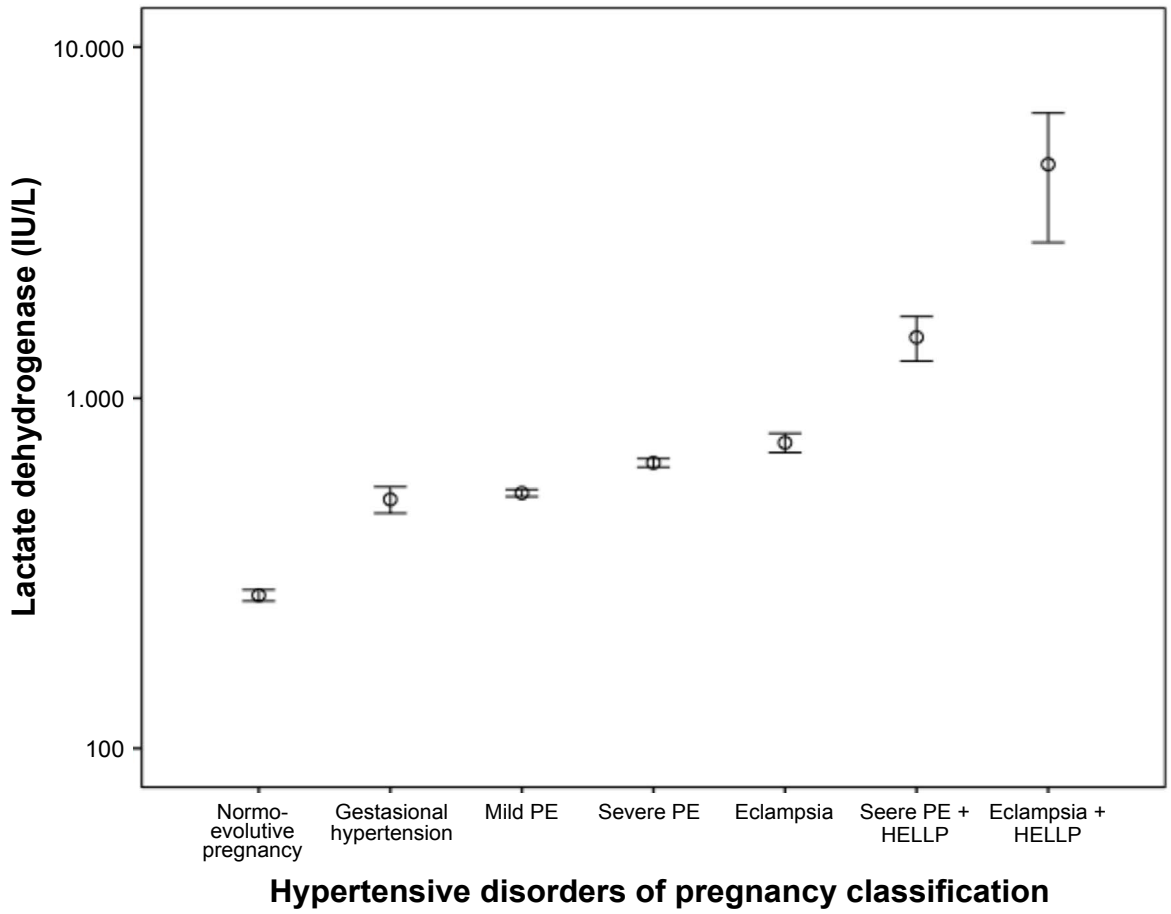

Figure 2: Correlation between HDP and LD concentrations.

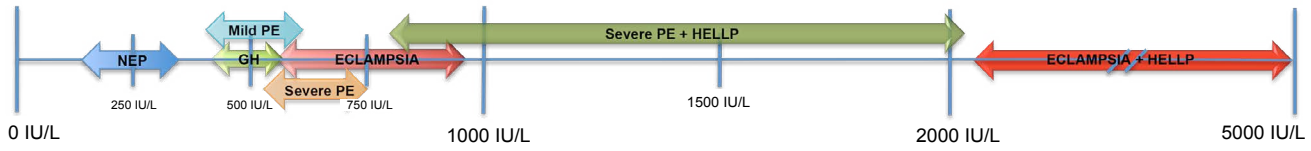

Figure 3: Baseline concentrations of LD in Mexican pregnant women with normo-evolutive pregnancy and HDP.

this study, and similar findings were reported first by Kuklina, et al. [20] in 2006, and recently by Cavazos-Rehg, et al. [21]. We also found that eclampsia was more frequent in first-time pregnant women unlike in any other HDP cases. The goal of monitoring symptoms and biochemical markers in patients with HDP is to appropriately time interventions and delivery while avoiding untoward maternal or neonatal complications. In contrast, LD has had an interesting journey, but today its clinical utility has been relegated to confirm hemolysis and serve as a tumor marker [22]. A study similar to ours was conducted by Qublan, et al. [15] and those authors found elevated LD levels at 111 IU/L in women with mild and severe PE, classifying three groups and LD values as follows: healthy pregnant (600 IU/L), mild PE (600-800 IU/L), and severe PE (800 IU/L), and they concluded that LD is indicative of cellular damage and dysfunction, and it can be used as a biochemical marker because it reflects the severity of the disease, occurrence of complications, and fetal outcome. In contrast, Jaiswar, et al. [22] conducted a study to evaluate LD as a biochemical marker for PE-eclampsia, and they analyzed 146 Indian women. They reported LD levels of 278.3 ± 119.2 IU/L in healthy pregnant women, and this value is similar to that found in our study (n = 379 and LD = 274 ± 100.7 IU/L). Furthermore, they concluded that LD levels had a significant association with various maternal and fetal outcomes in patients with PE and eclampsia. Other biomarkers in serum, such as soluble fms-like tyrosine kinase-1 (sFLt-1), vascular endothelial growth factor (VEGF), soluble intracellular adhesion molecule-1 (sICAM-1) and, soluble vascular intracellular adhesion molecule-1 svICAM-1, were varied and could not be pooled. To date, the literature on serum biomarker involvement in angiogenesis or inflammation after HDP has been inconsistent, with reports of lower [23] or higher [24] levels in women with PE. In fact, controversy exists because while Myatt, et al. [25] did not find that assessment of changes in angiogenic markers alone from the first or second trimester can improve predictive power, Widmer, et al. [26] also concluded in a multicenter study that angiogenic biomarker tests performed at ≤ 20 weeks gestation did not perform well enough in predicting PE for incorporation into current practice. However, these serum biomarkers, which are responsible for the initial endothelial insult during early pregnancy and are more expressed in placental tissue than in the endothelium, may not be detected at elevated levels after the index pregnancy. To clarify the confusing data regarding angiogenic factors and the need to obtain a better interpretation of traditional biomarkers, we propose LD values as indicative of cellular damage and endothelial dysfunction in HDP, and LD can be used as an effective biochemical marker because it reflects the severity of the disease, occurrence of complications, and fetal outcomes [15] in the Mexican population.

HDP diagnosis and classification are the main goals in pregnant women with high blood pressure, and the correct evaluation of clinical and laboratory parameters is important to achieve these goals. In this study, we showed that LD concentrations in HDP are predictive of adverse maternal outcomes and, we propose new LD reference values to classify HDP based on serum LD concentration and established a baseline of LD concentrations based on HDP severity. Currently, the basic biochemical tests for LD remain in effect to diagnose and classify HDP, and their use continues to provide valuable information for diagnosis and classification of pregnant women with HDP.

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Disclosure

No potential conflict of interest was reported by the authors.

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ORIGINAL ARTICLE

Lack of Association between Mannose-binding Lectin 2 Codons 54 and 57 Gene Polymorphisms and Cervicovaginal Infections in Mexican Women

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ABSTRACT

The mannose-binding lectin (MBL) 2 gene has an important function in the innate immune response and activation of the third pathway of the complement system. Some studies have assessed the association of the MBL2 gene polymorphisms with cervicovaginal infections (CVI); however, there is no information about this association in Mexican women. This study aimed to determine the association between the MBL2 codons 54 and 57 gene polymorphisms with CVI in a sample of Mexican women. Through a cross-sectional study, blood samples and cervicovaginal cultures were obtain from 354 women. MBL2 genotyping was performed by real-time polymerase chain reaction with Taqman probes. Of the 354 women studied, 128 (36.2%) had CVI and 226 (63.8%) were healthy. The frequencies of the C and T variants in codon 54 in women with CVI were 83% and 17%, respectively; whereas the frequencies of these variants in healthy women were 82% and 18%, respectively. The frequencies of variants C/C, C/T, and T/T in women with CVI were 68%, 31%, and 1%, respectively; whereas the frequencies of these variants in healthy women were 68%, 29%, and 3%, respectively. With respect to codon 57, the frequencies of variants C and T were identical in women with CVI and in healthy women (97% and 3%, respectively). The frequencies of variants C/C, C/T, and T/T were identical in women with CVI and in healthy women (94%, 6%, and 0%, respectively). We conclude that MBL2 codons 54 and 57 gene polymorphisms do not associate with CVI in Mexican women. (*Int J Biomed Sci* 2017; 13 (2): 79-83)

Key words: Cervicovaginal infections; MBL2 gene; codon 54; codon 57

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INTRODUCTION

Cervicovaginal infections (CVI) are a group of gynecological entities characterized by replacement of normal vaginal flora with infectious agents including virus, bacteria, fungi and protozoa (1, 2). CVI occur in women of any age and are one of the most important causes of medical consultations in primary healthcare centers (3, 4). It is estimated that 90% of CVI are caused by three groups of pathogens: a) anaerobic bacteria, mainly *Gardnerella vaginalis* leading to bacterial vaginosis; b) yeasts of the *Candida spp* genus leading to vulvovaginal candidiasis; and c) the protozoan parasite *Trichomonas vaginalis* (5-7). The innate immune system represents the first line of defense against infectious agents leading to an immediate response through several effector mechanisms that recognize and remove pathogens, and activate the adaptive immune system (8, 9). The mannose-binding lectin (*MBL*) 2 is a protein codified by a gene located on chromosome 10q11.2 (10, 11). This serum lectin is synthetized by the liver and it is released to the blood stream during the innate immune response against virus, bacteria, yeasts, and parasites (12). The *MBL* binds cell surface carbohydrates of pathogens mediating opsonization either directly or through complement activation by the lectin pathway (13). Low or deficient concentrations of MBL in serum are mainly due to single nucleotide polymorphisms of exon 1 of MBL2 gene (14). MBL2 codons 54 and 57 gene polymorphisms (variant allele O; wild-type allele designated as A) are denoted as B and C, respectively (15). These point mutations result in amino acid substitutions in the collagen region: in codon 54 (GGC->GAC, Gly->Asp, allele B), and in codon 57 (GGA->GAA, Gly->Glu, allele C) (16-18). Several studies have reported the association of MBL2 polymorphism with CVI (19-21). However, there is not any report about this association in Mexican population.

MATERIALS AND METHODS

Selection and description of participants

Through a cross-sectional study, 354 women attending consultations in the Family Healthcare Department in the Institute for Scientific Research of the Juárez University of Durango State in Durango City, Mexico were examined. Women were enrolled in the study from November 2014 to June 2016. Inclusion criteria for enrollment were: 1) age 17 years and older; 2) sexually active; and 3) who voluntarily accepted to participate in the study. Exclusion criteria were: 1) pregnant women, 2) women during menstrual period; 3) women under treatment for CVI in the last 10 days (including vaginal ovules, creams, or douching); 4) women with a recent miscarriage or postpartum; 5) history of hysterectomy; 6) suffering from autoimmune or systemic diseases; and 7) under treatment with immunosuppressive agents or antibiotics.

Technical information

Cervicovaginal secretions from participants were obtained with sterile cotton swaps and placed into routine culture media (chocolate agar, blood agar, and Thayer-Martin agar). In addition, a direct microscopic examination of the cervicovaginal secretions for the presence of pathogens was performed. Cervicovaginal secretions were also tested for the presence of amines, and examined using the Gram stain.

DNA was obtained from whole blood of participants by the QIAamp DNA Blood Mini Kit (QIAGEN) following the instructions of the manufacturer. The yield DNA concentration and purity were measured by the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Germering, Germany). Genotyping was performed using a real-time PCR equipment (StepOne, Applied Biosystems, Carlsbad, CA, USA) with TaqMan probes (codon 54: rs1800450; codon 57: rs1800451; Life Technologies, Australia). Typical reactions to a final volume of 20 µl consisted of 10 ng of genomic DNA, 0.625 µl TaqMan SNP genotyping assay, and 5.0 µl of genotyping master mix. Amplification was performed at 60°C for 30 seconds and 95°C for 10 minutes followed by 40 cycles of 92°C for 15 seconds and 60°C for 1 minute, and a final step of 60°C for 30 seconds.

Ethics aspects

This project was approved by the Ethics Committee of the Institute for Scientific Research of the Juárez University of Durango State, Mexico. An informed consent was obtained from all participants.

Statistics

Statistical analysis was performed using the software SPSS version.15.0. Allelic, genotypic and haplotypic frequencies were calculated with the aid of the software SNPStats, and odds ratio (OR) and 95% confidence interval (CI) were calculated. *P* values less than 0.05 were considered statistically significant.

RESULTS

One hundred and twenty-eight (36.2%) of the 354 women studied had CVI. Of them, 72 (20.3%) had bacterial vag-

inosis, 49 (13.8%) vulvovaginal candidiasis, and 7 (2.0%) trichomoniasis. Two hundred and twenty-six (63.8%) women were healthy. Mean age of women was 36.4 ± 10.3 (range: 17-67) years. Mean age at first sexual relation was 19.3 ± 3.8 (range: 11-40) years. Mean number of sexual partners was 3.0 ± 4.2 (range: 1-50). Mean number of sexual intercourses a month was 6.9 ± 5.6 (range: 0-40), and the median number of miscarriages was 0 (range 0-7). The frequencies of the C and T variants in codon 54 in women with CVI were 83% and 17%, respectively; whereas the frequencies of these variants in healthy women were 82% and 18%, respectively. The frequencies of variants C/C, C/T, and T/T in women with CVI were 68%, 31%, and 1%, respectively; whereas the frequencies of these variants in healthy women were 68%, 29%, and 3%, respectively. No association between the C/C reference genotype, C/T genotype polymorphism (OR=0.98; 95% CI: 0.60-1.58), and T/T genotype (codominant, OR=4.65; 0.55-39.1) and CVI was found. Codon 54 polymorphisms had genotypic distributions consistent with Hardy-Weinberg equilibrium in women with CVI (P=0.19), and in healthy women (P=1.0). With respect to codon 57, the frequencies of variants C and T were identical in women with CVI and in healthy women (97% and 3%, respectively). The frequencies of variants C/C, C/T, and T/T were identical in women with CVI and in healthy women (94%, 6%, and 0%, respectively). No association between this polymorphism (C/C reference genotype, C/T genotype, OR=1.18; 95% CI: 0.45-3.09) and CVI was found. Codon 57 polymorphisms had genotypic distributions consistent with Hardy-Weinberg equilibrium in women with CVI (P=1.0), and in healthy women (P=1.0). A correlation of the allelic and genotypic frequencies and the clinical characteristics of the women studied is shown in Table 1. Results of the association analysis of codons 54 and 57 MBL2 haplotypes with CVI are shown in Table 2. No haplotypic association with CVI was found (P=0.74).

Table 1. Allelic and genotypic frequencies of codons 54 and 57 polymorphisms of MBL2 gene in the women studied

Diagnosis[a]	Codon	Genotype	Positive No. (%)	Allele	Positive No. (%)	P^b value
Vulvovaginal candidiasis (n=49)	MBL54	C, C	36 (73.5)	C	85 (86.7)	
		C, T	13 (26.5)			
		T, T	0	T	13 (13.3)	0.27
Bacterial vaginosis (n=72)	MBL54	C, C	47 (65.3)	C	118 (81.9)	
		C, T	24 (33.3)			
		T, T	1 (1.4)	T	26 (18.1)	0.95
Trichomoniasis (n=7)	MBL54	C, C	5 (71.4)	C	12 (85.7)	
		C, T	2 (28.6)			
		T, T	0	T	2 (16.6)	1.00
Controls (n=230)	MBL54	C, C	155 (67.4)	C	378 (82.1)	
		C, T	68 (29.6)			
		T, T	7 (3.0)	T	82 (17.8)	
Vulvovaginal candidiasis (n=49)	MBL57	C, C	47 (95.9)	C	96 (97.9)	
		C, T	2 (4.1)			
		T, T	0	T	2 (2.0)	1.00
Bacterial vaginosis (n=72)	MBL57	C, C	67 (93.1)	C	139 (96.5)	
		C, T	5 (6.9)			
		T, T	0	T	5 (3.4)	0.77
Trichomoniasis (n=7)	MBL57	C, C	7 (100.0)	C	14 (100.0)	
		C, T	0			
		T, T	0	T	0	1.00
Controls (n=230)	MBL57	C, C	217 (94.3)	C	447 (97.1)	
		C, T	13 (5.7)			
		T, T	0	T	13 (2.8)	

[a]Four women had more than one infection; [b]Compared to controls (Fisher exact test).

Table 2. Association between codons 54 and 57 haplotypes of MBL2 gene and cervicovaginal infections

Codon 54	Codon 57	Women with cervicovaginal infections (n=124)[a]	Controls (n=230)	OR (95% CI)[b]	*P* value
C	C	81	79.8	-----	----
T	C	16.2	17.4	1.16 (0.73-1.83)	0.53
C	T	2.8	2.5	1.29 (0.47- 3.53)	0.62
T	T	0	0.3	[c]	

General haplotypic association: *P*=0.74. [a]Cervicovaginal infections: bacterial vaginosis, vulvovaginal candidiasis, and trichomoniasis; [b]Age-adjusted; [c]Undefined because of cells with a 0 value.

DISCUSSION

Cervicovaginal infections represent a public health problem in Mexican women. These infections have a high morbidity, and their complications are important cause of mortality in women at reproductive age (22). The present study aimed to identify the possible influence of codons 54 and 57 polymorphisms of *MBL2* gene on the presence of CVI including bacterial vaginosis, vulvovaginal candidiasis, and trichomoniasis in Mexican women.

Genetic components, vaginal microbiota, and local immunity play not only an important role in the health of women but also may contribute to a higher susceptibility to certain infections (23, 24). MBL2 is a vaginal component that protects against repeated proliferation of atypical vaginal microflora (25). Polymorphisms of the structural region of MBL2 gene, especially codon 54 and in a minor extent codon 57, cause alterations in the mannose-binding lectin production (11). Several studies in women populations have found a significant association between recurrent vaginal infections, especially vulvovaginal candidiasis and bacterial vaginosis, with the presence of codon 54 polymorphisms of MBL2 gene (7, 9, 14, 20, 21, 26). However, this association was not found in our study. It is important to mention that recurrent vulvovaginal infections were not considered in the present study. Only acute infections were included in our study. With respect to codon 54, the genotypic frequencies of homozygotes and heterozygotes variants in our study were 73.5% and 26.5%, respectively. These frequencies differ slightly from other frequencies reported in women with vulvovaginal candidiasis. For instance, frequencies of homozygotes and heterozygotes variants in women in China were 66.6% and 33.3%, respectively (20); whereas, these frequencies in women in Brazil were 64.3% and 35.7%, respectively (9). No mutated homozygotes were found in our study nor in the Chinese and Brazilian studies. Very little is known about the association of MBL2 with parasitic infections (12, 27). In the present study, we examined the association of codons 54 and 57 polymorphisms in women with trichomoniasis; however, this infection was present in only 8 individuals and no association of this infection with the polymorphisms was found. The fact that allele B (T) was not associated with any case of cervicovaginal infections suggests that multiple factors other than local factors can be involved in the pathogenesis of vaginal infections.

No statistically significant difference in the frequencies of the codon 54 variants C/C, C/T, and T/T between women with CVI and healthy women was found (68%, 31%, and 1%; and 68%, 29%, and 3%, respectively). Types of CVI were not associated with codon 54 polymorphisms.

With respect to codon 57 polymorphisms, we did not find mutated homozygotes, only seven women with CVI were heterozygotes: two with vulvovaginal candidiasis and five with bacterial vaginosis. Of the healthy women, 13 were heterozygotes, and there was not association between this polymorphism and CVI. This finding is consistent with others reported in the literature (9).

In the present study, all four possible haplotypes were found. However, no association between these haplotypes and CVI was found. Linkage disequilibrium between codon 54 and codon 57 was detected (D'=0.9903, *P*=0.04). To the best of our knowledge, there are no reports in the literature about the link of these haplotypes with acute CVI. MBL2 polymorphisms have been associated with susceptibility to tubal factor infertility (28).

One limitation of the present study was that we did not measure the concentrations of MBL2 protein in vagina. Further studies to determine the association of MBL2 protein concentrations and codons 54 and 57 polymorphisms in Mexican women are needed.

We conclude that MBL2 codons 54 and 57 gene polymorphisms do not associate with CVI in Mexican women.

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ABBREVIATIONS

CI	Confidence interval
CVI	Cervicovaginal infections
MBL	Mannose-binding lectin
OR	Odds ratio

CONFLICT OF INTEREST

The authors declare that no conflicting interests exist.

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