Universidad Juárez del Estado de Durango

Colegio Duranguense de la Química A.C.

Afiliado a la

Federación Nacional de Químicos Clínicos, CONAQUIC, A.C.

DGP F-370

Afiliado a la

International Federation of Clinical Chemistry and Laboratory Medical, IFCC

Otorgan el presente

Reconocimiento

Dr. en C. Gerardo Alfonso Anguiano Vega

Por su participación como **Ponente** en las Conferencias de

"Hemocultivos, Diagnóstico Bacteriológico y Molecular"

Realizadas el día 30 de Abril del 2016, en la ciudad de Victoria de Durango, Dgo.

Durango, Dgo. a 29 de Abril del 2016

Quím. David Ozeda Soto
Presidente del Colegio
Duranguense de la Química A.C.

M. en C. Enrique Arturo Guerrero Sánchez
Secretario Académico del Colegio
Duranguense de la Química A.C.

M. en C. Martha Elia Muñoz Martínez
Directora de la Facultad de
Ciencias Químicas-UJED.

CENTRO DE BACHILLERATO TECNOLÓGICO
industrial y de servicios No. 130
Miguel Hidalgo y Costilla

VII COTELAC
VII CONGRESO
LABORATORISTA CLÍNICO

Otorga la Presente

Constancia

A: *Dr. Gerardo Alfonso Anguiano Vega*

Por su Destacada Participación como ponente en la Conferencia
"De la doble Hélice al Genoma Humano" : La medicina del nuevo milenio en hombros de gigantes.
en el 7mo. Congreso de la Carrera de Técnico Laboratorista Clínico.
Celebrado los días 21, 22 y 23 de Marzo del 2018.

Secretaria de Educación Pública
Centro de Bachillerato Tecnológico
Industrial y de Servicios No 130
"Miguel Hidalgo y Costilla"
Clave 10DCT0398M

M.E. Mucio Gabriel Moreno Irigoyen
DIRECTOR DEL PLANTEL

Durango, Dgo., 22 de Marzo de 2018

UNIVERSIDAD JUAREZ DEL ESTADO DE DURANGO
FACULTAD DE CIENCIAS QUIMICAS

UJED

CONSTANCIA

ANGUIANO VEGA GA; ORTIZ CORTEZ J, URTIZ ESTRADA N, MENDOZA HERNANDEZ JM, BARRAGÁN HERNANDEZ M.

Por la exposición del cartel:

"Análisis del uso de anticonceptivos como factor de riesgo para a la infección por diferentes genotipos de VPH en el estado de Durango"

1ra JORNADA ACADÉMICO - CIENTÍFICA

En el marco del XXXIII Aniversario de la Facultad de Ciencias Químicas
del 25 al 29 de Septiembre de 2017

D.C. Eda Guadalupe Ramírez Valles
Directora

DIRECCION

D.C. Marcelo Barraza Salas
Secretario Académico

SECRETARIA ACADÉMICA

UNIVERSIDAD JUÁREZ DEL ESTADO DE DURANGO
FACULTAD DE CIENCIA QUIMICAS
LABORATORIO DE BIOMEDICINA MOLECULAR

ANALISIS DEL USO DE ANTICONCEPTIVOS COMO FACTOR DE RIESGO A LA INFECCION POR DIFERENTES GENOTIPOS DE VPH EN EL ESTADO DE DURANGO

ANGUIANO VEGA G. ALFONSO.[1]*; ORTIZ CORTEZ JAQUELIN.[1], URTIZ ESTRADA NORMA.[1], MENDOZA HERNANDEZ J.MANUEL.[1], BARRAGÁN HERNANDEZ MARICELA.[2] * e-mail responsable: gerardo.anguiano@ujed.mx

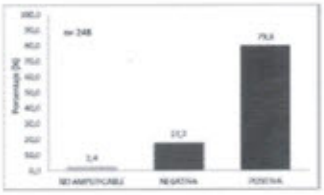
[1] LABORATORIO DE BIOMEDICINA MOLECULAR, FACULTAD DE CIENCIAS QUIMICAS , DGO UJED. [2]CLINICA DE DISPLASIAS, HOSPITAL MATERNO INFANTIL DE ESTADO DE DURANGO, SSED.

Introducción:

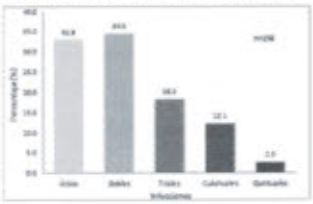
El cáncer cérvicouterino (CaCU) es la tercera causa de muerte por cáncer en mujeres mexicanas y la segunda en el mundo. El agente etiológico del CaCU es la infección por virus de papiloma humano (VPH); Dentro de los más de 200 genotipos identificados a la fecha existe un grupo de ellos considerado como VPH de alto riesgo a CaCU. Dentro de los factores de riesgo reconocidos para el desarrollo de displasias y CaCU el uso prolongado de anticonceptivos hormonales muy de los mas importantes.

Objetivo:

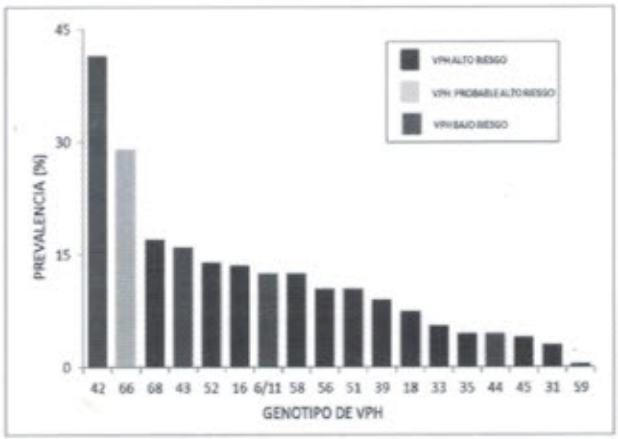
Analizar la asociación entre el uso de anticonceptivos hormonales y el incremento en el riesgo a presentar lesiones intraepiteliales o CaCu respecto a los genotipos virales infectantes



PORCENTAJE DE INFECCIÓN POR VPH



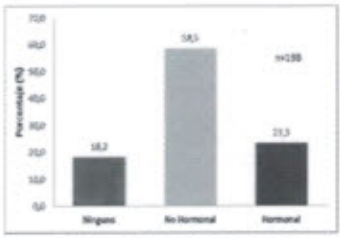
PORCENTAJE DEINFECIONES UNICAS Y MULTIPLES TIPOS DE VPH



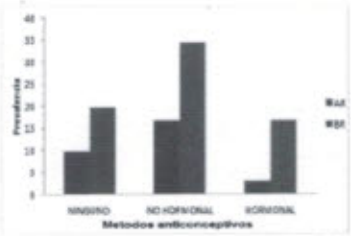
PREVALENCIA DE GENOTIPOS VIRALES DE VPH EN DURANGO

Material y Métodos.

Se obtuvieron raspados cervicales de 248 mujeres atendidas en el HMID. La técnica molecular utilizada fue PCR Múltiple Anidada, que consta de la tipificación por PCR mediante una doble amplificación de los genes E6 y E7 del VPH. Se elaboró una hoja informativa de factores de riesgo y diagnostico colposcópico que nos permitió realizara las pruebas de asociación de uso de anticonceptivos hormonales contra tipos virales y grados de lesión colposcópica.



FRECUENCIA DE USO DE TIPOS DE ANTICONCEPTIVOS



PREVALENCIA DE GENOTIPOS ALTO RIESGO Y BAJO RIESGO DE VPH CONTRA TIPOS DE ANTICONCEPTIVOS UTILIZADOS

ANALISIS ESTADISTICO POR CHI CUADRADA (χ^2) PARA LA RELACION DE LA INFECCIÓN DE VPH Y EL TIPO DE ANTICONCEPTIVO UTILIZADO

Anticonceptivo	VPH Positivo	VPH Negativo		χ^2calc.	χ^2tab.	P(0.05)
Ninguno	55	16		9.25	5.99	Sig
No Hormonal	103	15				
Hormonal	41	19				
Anticonceptivo	VPH-AR	VPH-BR	VPH AR-BR	χ^2calc.	χ^2tac.	P(0.05)
Ninguno	10	21	25	2.64	9.48	N.S.
No Hormonal	17	36	50			
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Inyectable	19	9		1.19	5.99	N.S.
Oral	16	9				
Otro	6	1				

Conclusión.

Los genotipos circulantes mas prevalentes en mujeres con lesiones intraepiteliales de bajo y alto grado o CaCU fueron los tipos VPH42, VPH66 y VPH68. Se confirma la relación entre infecciones por VPH de Alto Riesgo o Bajo Riesgo y el uso de anticonceptivos hormonales. No existe relación entre la infección de VPH Alto Riesgo o Bajo Riesgo con el tipo de anticonceptivo utilizado. No existe relación entre la infección por VPH y el método anticonceptivo hormonal utilizado.

Asociación Mexicana de Infectología y Microbiología Clínica, A.C.

CENTEOTL

Otorga la presente

CONSTANCIA

por su presentación de Trabajo Libre

Cartel C2

Título ANÁLISIS DEL USO DE ANTICONCEPTIVOS COMO FACTOR DE RIESGO A LA INFECCIÓN POR DIFERENTES GENOTIPOS DE VPH EN EL ESTADO DE DURANGO

Autores ANGUIANO VEGA G. ALFONSO.1*; ORTIZ CORTEZ JAQUELIN.1, URTIZ ESTRADA NORMA.1, MENDOZA HERNANDEZ J.MANUEL.1, BARRAGÁN HERNANDEZ MARICELA.2. 1. LABORATORIO DE BIOMEDICINA MOLECULAR, FACULTAD DE CIENCIAS QUÍMICAS (DURANGO) UNIVERSIDAD JUÁREZ DEL ESTADO DE DURANGO. 2. CLÍNICA DE DISPLASIAS HOSPITAL MATERNO INFANTIL (HMID) SSD DURANGO. DURANGO, MÉXICO

Durante el ***XLII Congreso Nacional de Infectología y Microbiología Clínica*** del 24 al 27 de Mayo de 2017 en el Centro de Convenciones William O. Jenkins, Puebla, Puebla.

Noris M. Pavia Ruz.

Dra. Noris Marlene Pavia Ruz
Presidente AMIMC A.C.

Dra. Patricia Cornejo Juárez
Secretario Académico

Categoría C No. C2

UNIVERSIDAD JUÁREZ DEL ESTADO DE DURANGO
FACULTAD DE CIENCIA QUIMICAS
LABORATORIO DE BIOMEDICINA MOLECULAR

ANALISIS DEL USO DE ANTICONCEPTIVOS COMO FACTOR DE RIESGO A LA INFECCION POR DIFERENTES GENOTIPOS DE VPH EN EL ESTADO DE DURANGO

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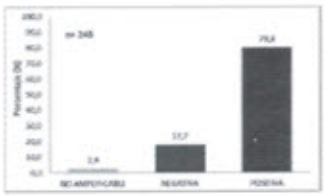

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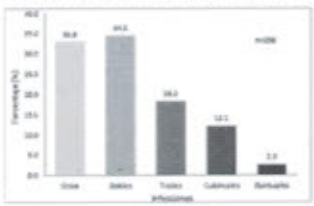

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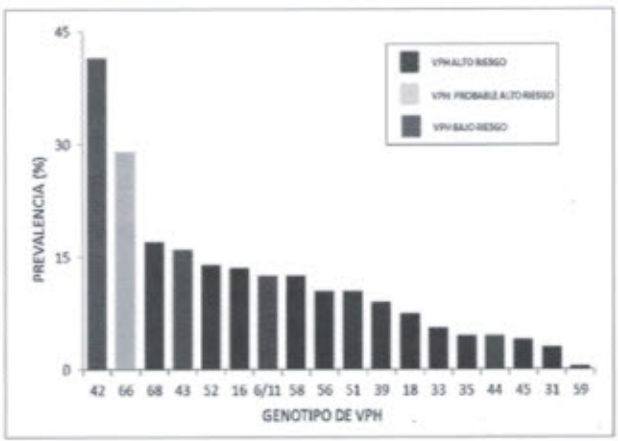

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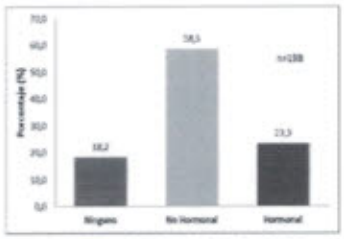

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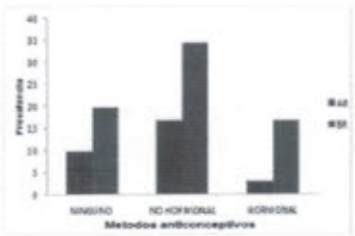

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SEP
SECRETARÍA DE EDUCACIÓN PÚBLICA

CENTRO DE BACHILLERATO TECNOLÓGICO
industrial y de servicios No. 130
Miguel Hidalgo y Costilla

CENTRO DE BACHILLERATO TECNOLÓGICO C.B.T.i.s. 130 industrial y de servicios No. 130

Constancia

A: *D. en C. Gerardo Alfonso Anguiano Vega*

Por su Destacada Participación como ponente en la Conferencia
"Veneno de alacrán: impacto en la salud y fuente de fármacos y productos biotecnológicos de alta relevancia"
en el 6to. Congreso de la Especialidad de Laboratorio Clínico.
Celebrado los días 26, 27 y 28 de Abril del 2017.

Durango, Dgo., 27 de Abril de 2017

M.E. Mucio Gabriel Moreno Irigoyen
DIRECTOR DEL PLANTEL

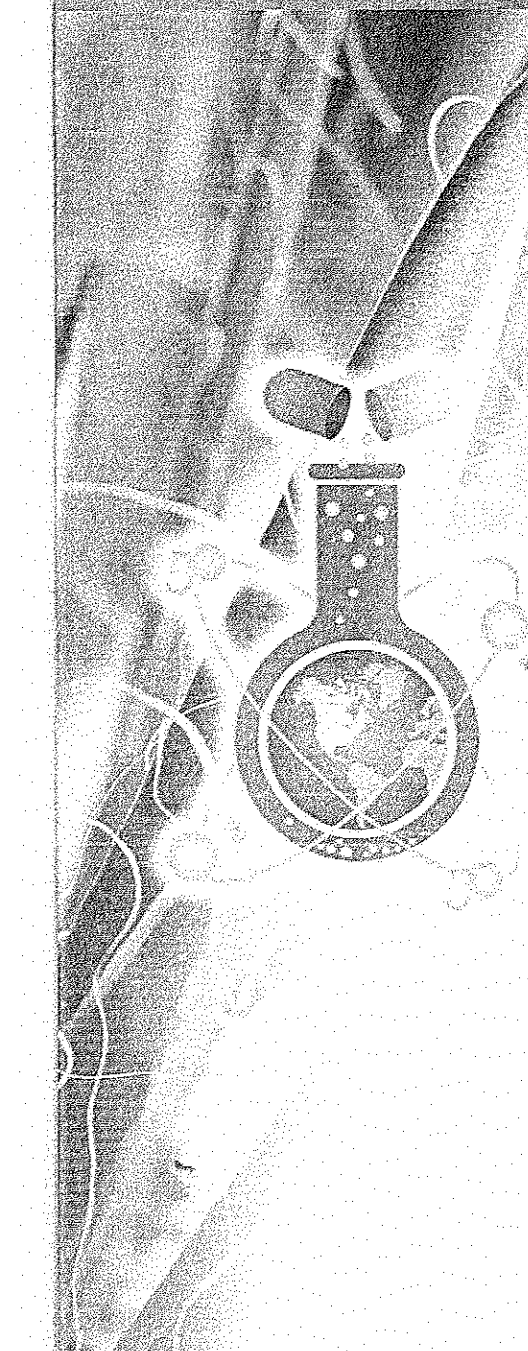
ESTADOS UNIDOS MEXICANOS

Secretaría de Educación Pública
Centro de Bachillerato
Tecnológico Industrial
y de Servicios No. 130
Durango, Dgo.

sems

DGETI

REVISTA MEXICANA DE CIENCIAS FARMACÉUTICAS



I Congreso Nacional de Ciencias Farmacéuticas

Zapotlán Sedeño

3 a 7 de Septiembre de 2017

"Hacia nuestro aseguramiento de la Calidad"

RESÚMENES
de Trabajos Libres

50 AÑOS 1967-2017

Volumen 48 • Suplemento 1 • Septiembre 2017

Volumen 48 • Suplemento 1 • Septiembre 2017

REVISTA MEXICANA DE CIENCIAS FARMACÉUTICAS

CONTENIDO

Áreas

TRABAJO LIBRE - QFA0009

Cribado *in silico* en el dominio N de la proteína E1 del VPH16

María Leticia Saucedo Mendiola, Estela Ruíz Baca, Karina Corral Pérez

Universidad Juárez del Estado de Durango

Introducción. La infección por virus del papiloma humano (VPH) es el principal agente causal del cáncer cervicouterino, el cual tiene una incidencia global estimada de 527,624 nuevos casos y 265,672 muertes al año, siendo la segunda causa de mortalidad femenina por cáncer a nivel mundial. Actualmente no existe ningún tratamiento antiviral para la infección de VPH. Un enfoque en la obtención de nuevos fármacos es el desarrollo de inhibidores de proteínas que sean importantes en la patogénesis del virus. En este sentido, el pequeño genoma de ADN del VPH, se mantiene activo como un episoma multicopia en el núcleo de las células epiteliales infectadas. Este proceso es dependiente de la replicación del genoma viral por las proteínas virales E1 y E2 y la maquinaria de replicación de ADN del huésped.

Objetivo. búsqueda mediante reconocimiento molecular *in silico* de moléculas que inhiban la replicación del VPH tipo 16.

Metodología. Se construyó y validó un modelo tridimensional del dominio N terminal de la proteína E1 del VPH tipo 16 mediante modelado por homología. Posteriormente se realizó un cribado virtual, la biblioteca de moléculas pequeñas utilizada fue la colección Hit Finder de la compañía Maybridge. Primeramente, se seleccionaron las moléculas con mayor energía de unión y de estas se seleccionaron 6 moléculas en base al modo de unión y las interacciones en la región reguladora N-terminal de la proteína E1 del VPH tipo 16.

Resultados. Las moléculas seleccionadas fueron la 9022, 12542, 11749, 9022, 5766 y 4177 con un potencial de unión de -8.7081, -8.3425, -7.9740, -7.7876, -7.1932 y -7.0512 kcal/mol respectivamente. Las moléculas seleccionadas contienen un grupo amino y a través de este forman puentes de hidrógeno con el Asp40 o el Asp 42 y la Thr 43.

Conclusiones. Mediante reconocimiento molecular se encontraron los primeros 5 inhibidores potenciales de la actividad de replicación del VPH16. Estas moléculas interaccionaron con el Asp42 y la Thr 43, residuos altamente conservados entre los diferentes tipos de VPH.

TRABAJO LIBRE - QFA0010

Cribado *in silico* de moléculas antagónicas de la interacción E6/E6AP del VPH tipo 16

María Leticia Saucedo Mendiola, María del Socorro Vázquez Mendieta, Gerardo Alfonso Anguiano Vega

Universidad Juárez del Estado de Durango

Introducción. La infección por Virus del Papiloma Humano (VPH) es el principal agente causal del Cáncer Cervico-uterino (CaCu), el cual tiene una incidencia global estimada de aproximadamente 527,624 nuevos casos y 265,672 muertes al año. El desarrollo de la neoplasia cervical se explica por la acción de las oncoproteínas virales E6 y E7. Estas tienen la capacidad de inmortalizar y transformar queratinocitos. La proteína p53 se activa cuando el ADN celular sufre algún daño, induciendo su reparación o su apoptosis. La degradación de p53 tiene como consecuencia la desregulación del ciclo celular. La acción clave de E6 en los VPH de alto riesgo está en inhibir la función de p53, en unión con la proteína celular asociada a E6 (E6AP).

Objetivo. Cribado *in silico* de moléculas antagónicas de la interacción E6/E6AP del VPH tipo 16.

Material y método: La estructura tridimensional de la oncoproteína E6 del virus del papiloma humano tipo 16 se obtuvo del PDB (3D1VZN), posteriormente se seleccionó la cavidad en donde se realizará el reconocimiento molecular, esta cavidad es el sitio de unión a la proteína E6 asociada. La biblioteca de moléculas utilizada fue la colección Hit FinderTM de la compañía Maybridge

Resultados. Las moléculas con mayor potencial de unión fueron la 2108, 9130,14085, con un potencial de unión de -14.4428, -13.5956, -13.5668, kcal/mol respectivamente. Mediante un análisis de las interacciones, la molécula 2018 presenta interacciones mediante puentes de hidrógeno con Ser 78, Lys 79 y Tyr 83, la molécula 14085 interacciona con Lys 79 y Tyr 83 mientras que la molécula 9130 presenta un solo puente de H con la Tyr 99.

Conclusiones. Se encontraron 3 moléculas que pueden ser antagónicas de la interacción E6/E6AP del VPH tipo 16.

SEP
SECRETARÍA DE EDUCACIÓN PÚBLICA

Instituto Politécnico Nacional
"La Técnica al Servicio de la Patria"

CIIDIR IPN DURANGO

Instituto Politécnico Nacional
Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional Unidad Durango
Sociedad Latinoamericana de Farmacogenómica y Medicina Personalizada

SOCIEDAD LATINOAMERICANA DE FARMACOGENÓMICA Y MEDICINA PERSONALIZADA

2° Congreso Latinoamericano de Farmacogenómica y Medicina Personalizada
Durango, Dgo., México
"Corazón de Latinoamérica"

Otorgan la presente

CONSTANCIA

a: SANTILLÁN SIDÓN A. PATRICIA; VÁZQUEZ BOUCARD CELIA; PÉREZ MORALES REBECA; ANGUIANO VEGA GERARDO A.; RUIZ BACA ESTELA; OLIVAS CALDERÓN EDGAR H.

Autores del trabajo:

"Relación entre la Actividad de Glutatión S- Transferasa GST y Presencia de Polimorfismos de los Genes *GSTM1, GSTT1* en Trabajadores Mexicanos Expuestos Ocupacionalmente a Plaguicidas" Clave:**TXG802**

Por su participación en la sesión de posters en el

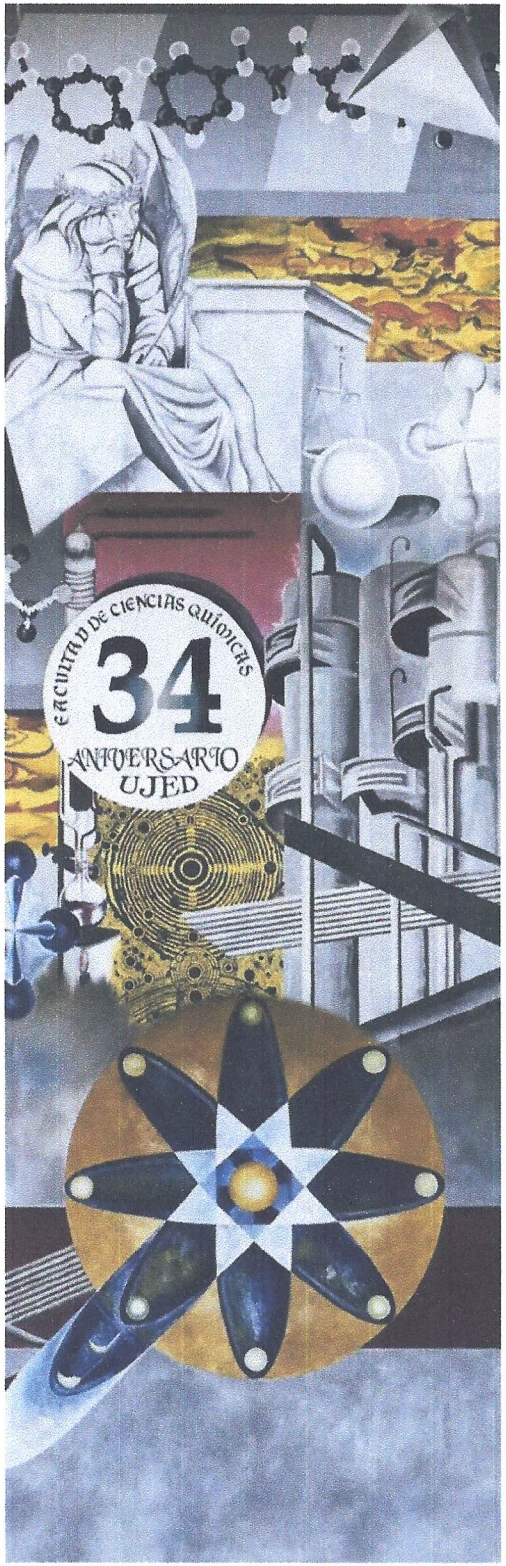
2do Congreso Latinoamericano de Farmacogenómica y Medicina Personalizada

Realizado en Durango, Dgo., México, del 25 al 28 de octubre de 2017

Dr. Eduardo Sánchez Ortíz
Director de CIIDIR IPN Unidad Durango

Profr. Dr. Luis Quiñones
Presidente de la SOLFAGEM

Dr. Ismael Lares-Asseff
Presidente del Comité Organizador



UJED

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UJED

Constancia

A: Santillán Sidón A. Patricia, Vázquez Boucard Celia, Pérez Morales Rebeca, Ruiz Baca Estela, Olivas Calderón Edgar H, Anguiano Vega Gerardo A.

Por obtener el primer lugar en el área de investigación en salud en la

modalidad de cartel con el trabajo titulado:

"Análisis de biomarcadores de exposición, efecto y susceptibilidad genética en trabajadores expuestos ocupacionalmente a plaguicidas"

II JORNADA ACADÉMICO - CIENTÍFICA

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Secretario Académico

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INSTITUTO DE INVESTIGACIÓN CIENTÍFICA

Otorga la presente:

Constancia

A ***Santillán Sidón A. Patricia, Vázquez Boucard Celia, Pérez Morales Rebeca, Ruiz Baca Estela, Anguiano Vega Gerardo A., Olivas Calderón Edgar H.***

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Atentamente

" Por mi raza hablará el espíritu "

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Broadening the neutralizing capacity of a family of antibody fragments against different toxins from Mexican scorpions

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ABSTRACT

New approaches aimed at neutralizing the primary toxic components present in scorpion venoms, represent a promising alternative to the use of antivenoms of equine origin in humans. New potential therapeutics developed by these approaches correspond to neutralizing antibody fragments obtained by selection and maturation processes from libraries of human origin. The high sequence identity shared among scorpion toxins is associated with an important level of cross reactivity exhibited by these antibody fragments. We have exploited the cross reactivity showed by single chain variable antibody fragments (scFvs) of human origin to re-direct the neutralizing capacity toward various other scorpion toxins. As expected, during these evolving processes several variants derived from a parental scFv exhibited the capacity to simultaneously recognize and neutralize different toxins from *Centruroides* scorpion venoms. A sequence analyses of the cross reacting scFvs revealed that specific mutations are responsible for broadening their neutralizing capacity. In this work, we generated a set of new scFvs that resulted from the combinatorial insertion of these point mutations. These scFvs are potential candidates to be part of a novel recombinant antivenom of human origin that could confer protection against scorpion stings. A remarkable property of one of these new scFvs (ER-5) is its capacity to neutralize at least three different toxins and its complementary capacity to neutralize the whole venom from *Centruroides suffusus* in combination with a second scFv (LR), which binds to a different epitope shared by *Centruroides* scorpion toxins.

1. Introduction

The severity of envenomations caused by scorpion stings reported in Mexico and around the world (Chippaux, 2012) is associated with the presence of abundant and potent neurotoxins in the venom. Therefore, medical importance of these beta-neurotoxins relies on two key elements, abundance and toxicity reflected as their affectation of voltage-dependent sodium (Na_v) channels (Cestèle et al., 1998; Possani et al., 1999) that are present in the central and peripheral nervous systems and are responsible for the transmission of nerve impulses. The fatal effect of the toxins relates

Abbreviations: scFv, single chain antibody fragment; CDR, complementarity determining region; FW, framework region; V_L, light chain variable domain; V_H, heavy chain variable domain; *C. noxius*, *Centruroides noxius* Hoffmann; Cn, toxin from *C. noxius*; *C. suffusus*, *Centruroides suffusus*; Css, toxin from *C. suffusus*; *C. limpidus*, *Centruroides limpidus*; Cll, toxin from *Centruroides limpidus*; *C. tecomanus*, *Centruroides tecomanus*; Ct1a, toxin from *C. tecomanus*; LD_{50}, Median lethal dose.

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to the impairment of this transmission affecting some vital functions, which can lead to death.

The current antivenom applied in Mexico is produced by hyperimmunizing horses with the venoms from four venomous scorpion species. Serum antibodies are enzymatically processed to obtain $F(ab')_2$ type fragments (Dehesa-Dávila and Possani, 1994). The high homology shared by scorpion toxins (de la Vega et al., 2013; Rodríguez De La Vega and Possani, 2005), would favor the generation of cross reacting antibodies. This antivenom consisting of the whole content of cross reacting antibodies, is expected to be polyclonal and polyvalent. Its polyvalent character is revealed by the capacity to neutralize the whole venoms of at least 7 venomous scorpion species from Mexico: *Centruroides noxius, Centruroides limpidus, Centruroides suffusus, Centruroides tecomanus, Centruroides infamatus, Centruroides elegans*, and *Centruroides sculptures*.

Intensive research on scorpion venom components, has revealed that usually there are one to three major neurotoxins in each venom (Bahraoui et al., 1988; Clot-Faybesse et al., 1999; Martin et al., 1987; Possani et al., 1981; Ramírez et al., 1994; Zamudio et al., 1992). As already mention, medical importance of these beta-neurotoxins is based on their abundance and toxicity (lethality) (Possani et al., 1999; Rodríguez De La Vega and Possani, 2005). It has been shown that polyclonal antibodies are not essential for neutralizing the toxic effect of the lethal components of scorpion venoms. For example, in *C. noxius* venom the main toxin (Cn2) represents 6.8% of the whole venom and shows an LD_{50} of 0.25 μg/20 g mouse. A monoclonal antibody (BCF2) was able of neutralizing Cn2 toxin and the whole venom (Zamudio et al., 1992). Another example, corresponds to *Androctonus australis* scorpion venom which contains two main neurotoxins (AaHI and AaHII). This venom is neutralized by two fragments of antibodies directed against each of these toxins (Hmila et al., 2010). These examples and others documented in the literature (reviewed by Alvarenga et al., 2014; Rodríguez-Rodríguez et al., 2015) indicated that neutralization of the main toxins would be sufficient to neutralize the whole venom, meaning that it is possible to generate novel specific antivenoms with a few antibody fragments. Using a single antibody would be sufficient to neutralize each main beta-neurotoxin. However, the presence of two or more different neutralizing antibodies against each toxin would increase the therapeutic efficiency of the new antivenoms. In support of these observations, we have demonstrated that the neutralization of the venom of *Centruroides noxius* with two antibody fragments (LR and RU1), that recognize different epitopes on the Cn2 toxin is more efficient (Riaño-Umbarila et al., 2016). Smaller amounts of these scFvs mixed compared when injected separately, were sufficient to protect envenomed mice. Innovative antivenoms would have several advantages: a homogenous composition, limited batch-to-batch variation, better safety and efficacy. Furthermore, these products do not depend on the immune response of animals and the probability of occurrence of secondary effects associated with the heterologous origin of antivenoms might be minimized (Chippaux, 2012). Results reported by our group, indicate that single-chain variable fragments (scFv) of human origin capable of neutralizing the major beta-neurotoxins present in the venoms, represent a promising alternative to be used as a new antivenom against *Centruroides* scorpions stings (Riaño-Umbarila et al., 2016, 2013, 2011, 2005).

Initially, we isolated two non-neutralizing scFvs, 3F and C1 against toxin Cn2 from a human scFv library (Riaño-Umbarila et al., 2005). These parental antibody fragments were affinity matured by directed evolution and phage display. As a result of these processes, variants capable of neutralizing Cn2 toxin were generated. scFvs LR and RU1 were the best variants obtained from parental scFvs 3F (Riaño-Umbarila et al., 2011) and C1 (Riaño-Umbarila et al., 2016), respectively. It was shown that these fragments were capable of neutralizing a second toxin. scFv LR was able to neutralize Css2 toxin from the venom of *Centruroides suffusus*, while scFv RU1 neutralized Cll1 toxin from *Centruroides limpidus*. It is worth mentioning that these two scFvs recognize different epitopes. Both non-overlapping epitopes are supposed to be involved in the envenoming effects of the toxins (Riaño-Umbarila et al., 2016). The main aim of our group is to optimize the different antibody fragments derived from both families of scFvs (3F and C1) to develop variants being able of neutralizing the main toxins present in Mexican scorpion venoms. We suggest that a set of scFvs that bind to both epitopes will guarantee protection from envenoming. At the same time the polyclonal and polyspecific nature of the current antivenom would be imitated.

As mentioned above, scFv LR neutralizes the Css2 toxin. To further optimize the neutralization of this toxin, we sought to generate a second scFv derived from scFv C1 which would be able to neutralize Css2 toxin through a second epitope. At the same time, based on the high homology among *Centruroides* scorpion toxins, we would be broadening the neutralization capacity of the derived scFvs against other toxins. To achieve these goals, two mutagenesis processes were implemented in parallel; random and site-directed mutagenesis, using some of the variants derived from scFv RU1 taken as substrate. It is worth noting that scFv RU1 recognizes but does not neutralize Css2 toxin (data not shown). Two variants derived from scFv RU1 (scFvs RJI-1 and RJI-2) were subjected to *in vitro* maturation by means of directed evolution and semi-rational approaches. Generated libraries were subjected to several rounds of selection against Css2 toxin using phage display. Selected variants showed several changes that were combined, resulting in a new set of scFvs that were evaluated for their ability to bind to several toxins. The results of these experiments revealed that the incorporation of a few key mutations allowed to expand the binding capacity of the new scFvs toward various toxins; including Css2 (*C. suffusus*), Cn2 (*C. noxius*), Cll1, Cll2 (*C. limpidus*) and Ct1a (*C. tecomanus*). scFv ER-5 which was evolved toward Css2 toxin, was capable of neutralizing three different toxins. Structural analyses of the complexes of toxins with scFvs helped us to understand how this set of selected mutations significantly increased binding capacity, cross reactivity and affinities of the new variants against several toxins.

2. Experimental procedures

2.1. Venom preparation

The scorpion venoms used in this work were obtained from *Centruroides suffusus*, *Centruroides noxius*, *Centruroides limpidus* and *Centruroides tecomanus*. Fresh venom was obtained from individuals of each species by electrical stimulation. The samples were diluted in bidistilled water and centrifuged at 14,000 rpm for 10 min at 4 °C. The insoluble material was discarded, and the toxin-containing supernatant was recovered and spectrophotometrically quantified ($\lambda = 280$ nm).

2.2. Purification of the toxins

The beta-neurotoxins were purified from the scorpion venoms using previously described methodologies. Css2 and Css4 were obtained from *C. suffusus* (Espino-Solis et al., 2011), Cll1 and Cll2 were obtained from *C. limpidus* (Alagón et al., 1988; Ramírez et al., 1994), Cn2 was obtained from *C. noxius* (Zamudio et al., 1992) and Ct1a was obtained from *C. tecomanus* (Olamendi-Portugal et al., 2005).

2.3. Construction of the scFv variants and the combinatorial mutations

To construct the initial variants, the DNA sequence of scFv RU1 was modified at positions N57S (V_H), Y110T (V_H), Y110N (V_H) and S164I (V_L) using specific oligonucleotides (DIR and REV, which flank the sequence of scFvs) (Riaño-Umbarila et al., 2005) (Table 1). For each mutation, a mega-primer was generated in a PCR reaction using a specific primer and the DNA from scFv RU1 as the template. The mega-primer containing the H110T and H110N mutations was combined with the mega-primer containing the S164I mutation. The product of this PCR reaction was included in a new PCR using the mega-primer containing the N57S mutation. All of the PCR products were purified from a preparative agarose gel. The final complete variant products were digested with the *Sfi*I and *Not*I enzymes and ligated into the p*Syn*1 plasmid, which had previously been digested with the same enzymes, to further characterize the soluble protein products or into phagemid p*Syn*2 to display the scFvs on a phage (M13). The ligation products were introduced into electro-competent *E. coli* TG1 cells. The DNA sequence of each variant was confirmed by DNA sequencing (Sequencer equipment model 3100 Applied BioSystems; Foster City, CA). This same procedure was used to construct the different variants described in this study.

2.4. In vitro maturation of the RU1 variants by directed evolution

The maturation process included the construction of a mutant library by random mutagenesis and its screening using three rounds of bio-panning. Error-prone PCR reactions were performed to construct the mutant library. This method allows us to achieve different mutation rates under appropriate conditions (Cadwell and Joyce, 1992; Leung et al., 1989). The DNA sequences used as templates correspond to the scFv RU1 variants RJI-1 and RJI-2. The primers were DIR and REV (Riaño-Umbarila et al., 2005). The PCR products were gel-purified, digested with the restriction enzymes *Sfi*I and *Not*I, and ligated into the p*Syn*2 phagemid that had previously been digested with the same enzymes to display the scFvs on a phage (M13). The ligation product was incorporated into *E. coli* TG1 electro-competent cells. The library was characterized and evaluated by bio-panning against the Css2 toxin as previously described (Riaño-Umbarila et al., 2005) with some modifications. The toxin concentrations were 2 μg ml^{-1} for the first bio-panning round and 1.5 μg ml^{-1} for the second and third rounds. For the second round, the phages were pre-incubated at 60 °C for 1 h before incubation with immobilized Css2 toxin. The unspecific and weak binding phages were eliminated by washing with 100 mM trimethylamine, followed by several washes with PBS-Tween 20. The scFv population from the third round was evaluated with a soluble protein ELISA as described previously (Riaño-Umbarila et al., 2005). The DNA from the selected scFvs was sequenced using the primers DIR and REV in an Applied BioSystems Sequencer model 3100 (Foster City, CA).

2.5. Maturation by semi-rational strategy

A theoretical binary complex of scFv RJI-2-Css2 was generated through *in silico* mutagenesis and molecular replacement in the crystallographic structure of the ternary complex scFv RU1-Cn2-LR scFv (PDB entry 4V1D) using the standard rotamer library of Coot (Emsley et al., 2010). In this structure, scFv LR was removed and the Css2 toxin obtained by NMR (PDB entry 2LI7) (Saucedo et al., 2012) was structurally superposed to the Cn2 toxin and coupled to an *in silico* mutagenized scFv RU1 resulting in the scFv RJI-2. The model was subjected to energy minimization using YASARA software (Krieger et al., 2009). The structural scrutiny was focused on the area of interaction between scFv RJI-2 and the Css2 toxin in which there are differences in some amino acid residues with respect to the Cn2 toxin. The residues that were modified in scFv RJI-2 were changed from S 57 (V_H) to R and K; K 65 (V_H) to R and Y; and I 236 (V_L) to W, F and L. The library was constructed with the mega-primer strategy using scFv RJI-2 as the template, the degenerate primers S57R/K, K65R/Y, and I 236W/F/L (Table 1) and the primers DIR or REV (Riaño-Umbarila et al., 2005). The library was characterized and evaluated using three bio-panning rounds against the Css2 toxin as previously described (Riaño-Umbarila et al., 2005). The concentration of the toxin immobilized into an immuno-tube was 2.3 μg ml^{-1} for the first round of bio-panning, and 2 μg ml^{-1} for the second and third rounds.

2.6. Antibody fragment expression and purification

The DNA segments that encoded the selected scFvs were subcloned into the p*Syn*1 vector to express the proteins. The recombinant vectors were used to electroporate the *E. coli* TG1 cells. The proteins were expressed and purified as previously described (Riaño-Umbarila et al., 2005). The protein concentrations were determined spectrophotometrically at $\lambda = 280$ nm. An extinction coefficient of 49765 and molecular weights: RJI-1; 28621.5, RJI-2; 28634, RJI-3; 28657.5, ER-1; 28563.5, ER-5; 28673 and ER-6; 28550.4 were used.

2.7. Comparison of the binding properties by surface plasmon resonance

The level of recognition of the scFv variants was determined using a biosensor that detects molecular interactions in real time (Biacore X, Uppsala, Sweden) at 25 °C. The Css2, Cn2, Cll1, Cll2 and Ct1a toxins were dissolved in 10 mM 2-(*N*-morpholino) ethanesulfonic acid (pH 6) and immobilized on cell 2 of a CM5 sensor chip using the amino coupling kit, reaching binding levels of 200–300 RUs. Cell 1 in the sensor chip without antigen was used as a control. The same concentrations of the scFv variants were used to evaluate the binding to each toxin. The samples were diluted in HBS-EP

Table 1
Primer sequences used to construct the variants. M = A/C, Y = C/T, R = A/G S = C/G and K = G/T.

Strategy	Template	Primers	Oligonucleotides
Construction of the RJI-1, RJI-2 and RJI-3 variants.	RU1	N57S	5′AGTATTTACTACCTCCACCATATGA3′
	RU1	S164I	5′CTTGTTCTGGATCCAGCTCC3′
	RU1	H110T	5′GCGCCGACTGGACCTTCG3′
	RU1	H110N	5′GCGCCGACTGGAACTTCG3′
Maturation of RJI-2	RJI-2	S57 R/K	5′AGTATTTMYTACYTCCACCATATG3′
	RJI-2	K65 R/Y	5′GCAGACTCCGTGARGGCCG3′
	RJI-2	I236 W/F/L	5′GGGATGACAGCCTGTKSGGTTATG3′
Construction of the combinatorial mutations	ER-4	N57R	5′AGTATTTCCTACCTCCACCATATGA′3

buffer (Biacore) and 100 μL of the scFvs were injected over the chip at a flow rate 40 or 50 μL min^{-1} with a delay of 500 s. The chip surfaces were regenerated with 10 mM HCl. The sensorgrams were corrected by subtracting the values from the reference flow cell and comparing the results using BIA-evaluation software version 3.1.

2.8. Surface plasmon resonance measurements

The kinetic constants of the scFvs were determined using previously described conditions (Riaño-Umbarila et al., 2011) with some modifications. Only the two scFvs that exhibited the best interactions with the toxins were evaluated twice. The scFvs that had been serially diluted in HBS-EP buffer (Biacore) were injected into chips containing the immobilized Css2, Cn2, Cll1, Cll2 or Ct1a toxins. Samples of 100 μL were injected over each chip at a flow rate 50 μL min^{-1}. The protein concentrations ranged from 1 nM to 200 nM for each assay. The delay phase lasted 600 s or 900 s. The kinetic constants were determined using the corresponding sensorgrams, which were corrected by subtracting the values from both the reference flow cell and the blank buffer injection. The Langmuir (1:1) model from BIA-evaluation software version 3.1 was used to determine the kinetic constants. Time of residence (T_R) of the scFvs on the different toxins was determined as the $1/k_{off}$ value expressed in minutes.

2.9. Competition assay

The competition for the binding of the scFvs ER-5, RU1 and LR to the Css2 toxin was performed as previously described (Riaño-Umbarila et al., 2016).

2.10. Neutralization assays with the toxins

All *in vivo* neutralization assays were performed in accordance with the guidelines of the Bioethics Committee of the Institute of Biotechnology from the National Autonomous University of Mexico. The *in vivo* neutralization tests were performed with the different scFvs and 3 toxins (Css2, Cn2 and Cll1). Control groups of 10 female CD1 mice weighing ~20 g were intraperitoneally injected with toxin amounts that were equivalent to 1 or 2 LD_{50} (Table 2). In the experimental group, the toxin was mixed with each scFv variant to prepare samples with various toxin:scFv molar ratios (1:10, 1:5, 1:2 or 1:1). The mixtures were pre-incubated for 30 min at room temperature (~25 °C) prior to injection. The animals were observed over a period of 48 h.

2.11. Neutralization assays with whole fresh venom from C. suffusus

The *in vivo* neutralization tests were performed using freshly prepared venom. Control groups of 10 female CD1 mice weighing ~20 g were intraperitoneally injected with venom amounts corresponding to 2 or 3 median lethal doses (LD_{50}). For the first neutralization assay, the venom was mixed with scFv ER-5 to prepare samples containing different toxin:scFv molar ratios. These ratios were calculated relative to the concentration of the Css2 toxin in the venom. The LD_{50} of the venom of *C. suffusus* is ~8.75 μg/20 g body weight of each mouse, where the Css2 toxin represents ~2.8% of the total toxic components (Martin et al., 1987). Similar to the first test, a combination of ER-5 and LR was used for the second neutralization assay. The mixtures of venom and scFv(s) were pre-incubated at room temperature for 30 min prior to their injection into the mice.

2.12. In silico mutagenesis and modeling

Theoretical binary complexes were generated through mutations *in silico* based on the crystallographic structure of the ternary complex scFv RU1-Cn2-LR scFv (PDB entry 4V1D) in which the structure of scFv LR was removed. The generated models were scFv ER-1 with the toxins Cn2, Css2, Cll1, Cll2 and Ct1a; and scFv ER-5 with Css2. The *in silico* mutagenesis was performed using the structure of RU1 as the template. The Cn2 toxin was mutated to generate the Cll1, Cll2 and Ct1a toxins. The differences between the scFv variants and toxins can be observed in Table 3A and B and Fig. 1 and 2. The models were generated using the standard rotamer library of Coot (Emsley et al., 2010) and subjected to energy minimization using YASARA software (Krieger et al., 2009). The electrostatic potential surfaces were calculated by solving the Poisson-Boltzmann equation included in the ccp4mg program version 2.7.3 (McNicholas et al., 2011). The graphical representations were also constructed with *CCP4mg* version 2.7.3 (McNicholas et al., 2011).

3. Results

Using different approaches of *in vitro* maturation such as directed evolution, site-directed mutagenesis and phage display, we sought to direct the recognition of the parental scFvs 3F and C1 (Riaño-Umbarila et al., 2005) towards several different scorpion beta-toxins. The high sequence similarity among Mexican scorpion toxins (Rodríguez De La Vega and Possani, 2005) (Fig. 1) and cross reactivity of some scFvs (Riaño-Umbarila et al., 2016, 2011) are the main elements that have contributed to achieve the aims of this study.

Maturation of scFv C1 by directed evolution against Cll1 and Cll2 toxins from scorpion *Centruroides limpidus* resulted in the generation of a set of important scFv variants (Table 3A). The main result was achieved from a combination of mutations that were integrated to generate scFv RU1, which exhibited the ability to neutralize Cll1 and Cn2 toxins and the whole venom of *C. noxious* (Riaño-Umbarila et al., 2016). Efforts aimed at generating variants from the parental scFv C1 that could neutralize Cll2 toxin had been unsuccessful. However, key mutations that would help to broaden toxin recognition were identified during these maturation processes. These mutations were mainly located at CDR2 and CDR3 of the heavy chain variable domain (V_H) and fewer changes were located in the light chain variable domain (V_L). Based on the observation that the scFv RU1 carries mutations that improved the recognition of different toxins, mutations N57S, Y110H/T/N and S164I were incorporated into the sequence of scFv RU1 to generate a new set of scFvs (Table 3A). Obtained variants, share the same mutations with the exception of the mutation at 110 position; RJI-1

Table 2
Values of the toxins median lethal doses.

Scorpion venom	Toxin	1 LD_{50} (μg/20 g of mouse)	Reference
C. suffusus	Css2	0.75	(Estrada et al., 2007)
C. noxius	Cn2	0.25	(Zamudio et al., 1992)
C. limpidus	Cll1	1.7	(Riaño-Umbarila et al., 2013)

Table 3
A) Mutations identified in scFvs derived from parental scFv C1 during the process of *in vitro* maturation. B) Mutations identified in the scFvs isolated during maturation process against Css2 toxin.

A)

scFv	VH										VL					Approach-toxin
	FW1	CDR2			FW3		CDR3				FW1	CDR1	FW3		CDR3	
C1	50	54	56	57	69	84	105	107	110	111	164	172	220	225	235	
	V	S	D	N	T	N	M	A	Y	F	S	T	S	D	L	
3H		G							H							D.E.–Cll1
202F		G	G				L									D.E.–Cll1
RU1		G	G				L		H							C.–Cll1-Cn2
3008C		G			I		L				I				P	D.E.–Cll2
3008C-I		G			I		L		T		I				P	D.E.–Cll2
3007A		G										A				D.E.–Cll2
3004F	A	G		K	I	Y	L	S	H		I				P	D.E.–Cll2
3010B		G			I		L		N	L				V	P	D.E.–Cll2
RU2J		G	G	S	I		L		N	L	I			V	P	D.E.–Cll2
RJI-1		G	G	S			L		T		I					C.
RJI-2		G	G	S			L		N		I					C.
RJI-3		G	G	S			L		H		I					C.

B)

scFv	VH							VL		Approach
	CDR2			FW3	CDR3			FW1	FW3	
C1	54	56	57	65	105	107	110	164	208	
	D	S	N	K	M	A	Y	S	D	
ER-1	G	G	S		L		T	I	G	D. E.
ER-2	G	G	S		L		N	I	G	C.
ER-3	G	G	R		L		N	I		D.E. and S-R.
ER-4	G	G	S	R	L		N	I		S-R
ER-5	G	G	R	R	L		N	I	G	C.
ER-6	G	G	S		L		N		G	C.

Approaches: D.E. Directed Evolution; C: Combinatorial, which means that scFvs were generated by combining mutations that were identified in the other scFvs. The residues that were incorporated into RJI-1, RJI-2 and RJI-3 are shown in bold; S-R.: Maturation using the semi-rational strategy.

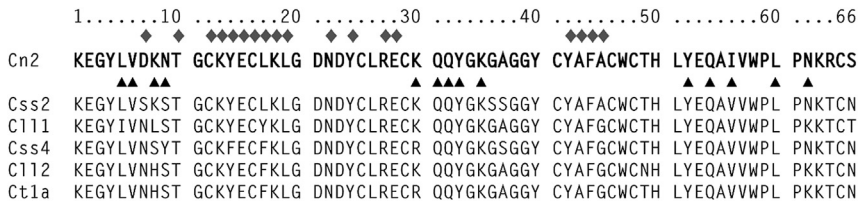

Fig. 1. Similarity of the different scorpion toxins from species of the *Centruroides* genus. The sequence alignment of the amino acid residues of the beta-neurotoxins exhibit a significant homology among the toxins (80–90%). The diamonds above the residues indicate the contacts between the Cn2 toxin and the scFv RU1 and the triangles below the residues indicate the contacts with the scFv LR, based on previous results (Riaño-Umbarila et al., 2016).

(T 110), RJI-2 (N 110) and RJI-3 (H 110) (Fig. 2 and Table 3A), which was identified during early maturation processes.

Binding capacity of the three RU1 variants (RJI-1, RJI-2 and RJI-3) to the various toxins was evaluated in a preliminary way by means of SPR. The respective sensorgrams revealed an improved binding towards Css2 and Cll2 toxins compared to scFv RU1 at the same concentration (Fig. 3Aand B). scFv RJI-2 shown a better binding response to Css2 toxin followed by RJI-1 and RJI-3 (Fig. 3A).

To generate a second set of improved scFvs capable of neutralizing the Css2 toxin, two simultaneous procedures of maturation were implemented: directed evolution and semi-rational approaches. The latter approach was performed with the help of a structural model which allowed to identify candidate mutations that would improve the recognition of Css2 toxin by the mutant scFvs.

3.1. Directed evolution

scFvs RJI-1 and RJI-2 were *in vitro* affinity matured in order to generate neutralizing variants against Css2 toxin. For this purpose, the genes that encode these scFvs were used as templates to construct a mutant library. A library of 3.3×10^7 transformants was subjected to three rounds of bio-panning using phage display. Two variants were isolated from the last round: ER-1 and ER-3 which contained D208G and N57R mutations, respectively (Fig. 2 and Table 3B). In ELISA assays, scFv ER-1 showed the highest binding to Css2 compared to scFvs ER-3 and RU1 (data not shown).

3.2. Site-directed mutagenesis

The theoretical model of the interaction between scFv RJI-2 and the Cn2 toxin showed that the side chain of residue K65 (V_H) at the interface establishes a hydrogen bond with residue N9 from Cn2 toxin and I236 (V_L) forms a hydrophobic interaction with toxin

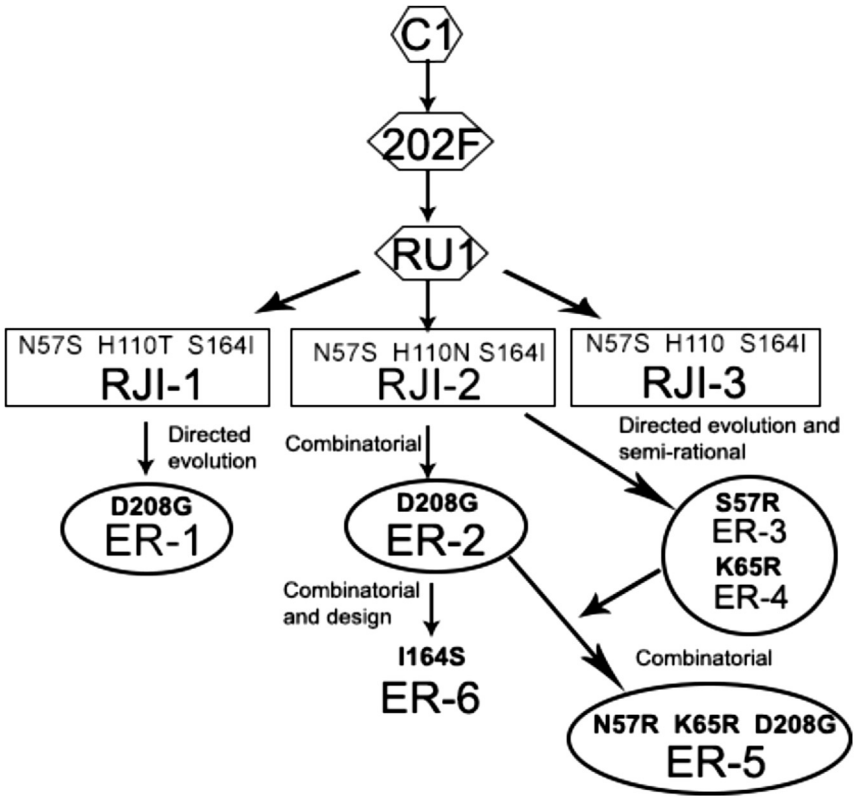

Fig. 2. Genealogy of scFvs derived from the parental scFv C1. Improved antibody fragments that were obtained in previous studies (hexagons) and the present work (rectangles and ovals) are shown. The name of the variants and mutations accumulated during the process of improving their neutralization capacity (top) are indicated. Rectangles include scFvs that were used for *in vitro* maturation under approaches indicated in the middle of the corresponding arrows. Ovals include mutant scFvs that resulted from these processes and were directed against Css2 toxin. scFv ER-6 was obtained by design and combinatorial approaches. This scFv was the best fragment that binds Cn2 toxin. The experimental approach that was used to obtain scFv ER-5 is also indicated.

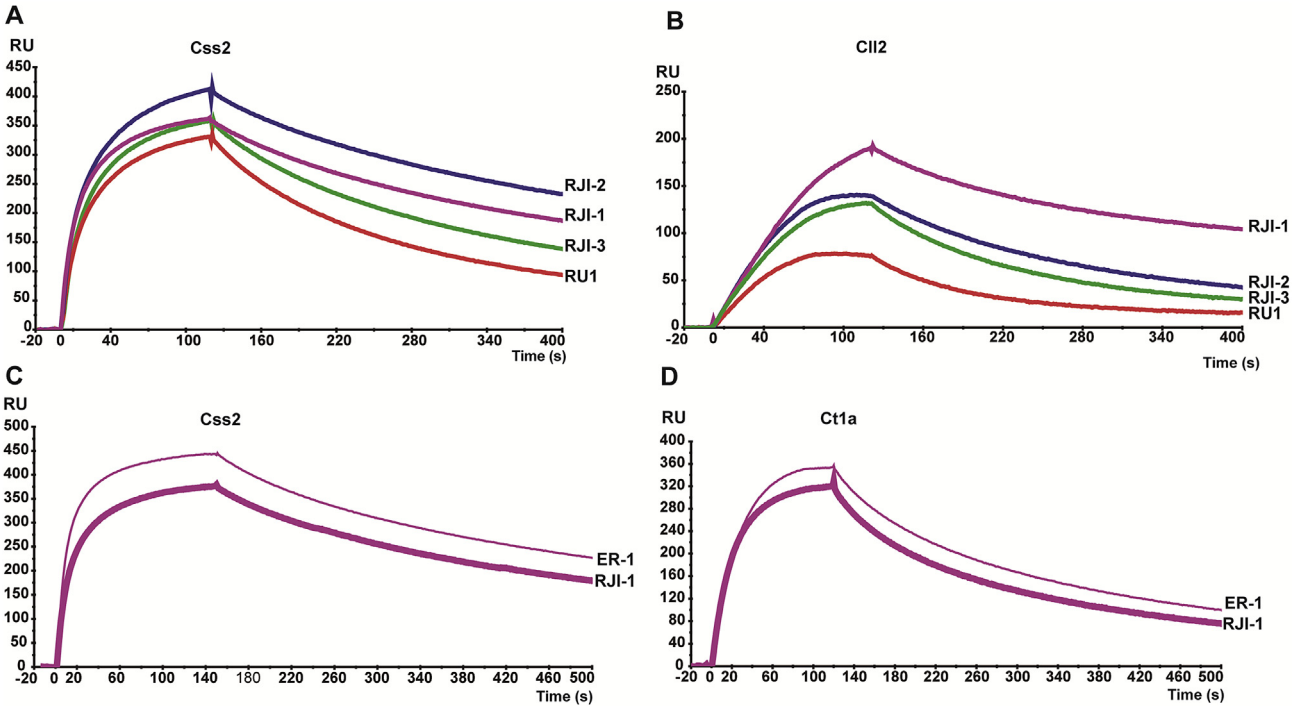

Fig. 3. Recognition of beta-toxins by different scFv variants. SPR sensorgrams correspond to the recognition of Css2 (**A**) and Cll2 toxins (**B**) by RJI-1, RJI-2 and RJI-3 compared to scFv RU1. Differences in the recognition of Css2 (**C**) or Ct1a (**D**) toxins can be observed in the variant with the D208G mutation (ER-1) compared to one which did not contain this mutation (RJI-1). Each of the scFvs were used at the same concentration in each panel: A: 200 nM, B: 100 nM, C: 200 nM, and D: 250 nM.

residue I56. Equivalent interactions occur in the RU1-Cn2 complex (Riaño-Umbarila et al., 2016). In Css2 toxin, the residues at these positions are S9 and V56 which carry comparatively smaller side chains (Fig. 1). When Cn2 is replaced with Css2 in the model, the increased distance between the interacting residues of Css2 toxin and scFv RJI-2 might cause a decrease in the strength of their interaction. To recover these contacts, the corresponding residues in the scFv were mutated to residues with longer side chains: K65 to R or Y and I236 to L, W or F. In addition to these two residues we decided to modify position S57 by K, based on the results of the maturation processes (see Table 3A). Serine (S) present in scFv RJI-2, is a non-charged residue while K is larger and charged. To evaluate the importance of the residue present in this position, in terms of the recognition to Css2 toxin, two additional mutations (R or G) were included. This set of mutations was combined with the mutations in the other two positions (65 and 236). To evaluate the effects of the combination of these changes a corresponding library was constructed using scFv RJI-2 as the template. A library of 3.8×10^8 variants was subjected to three rounds of bio-panning. Two main variants were isolated from the third round: scFvs ER-3 and ER-4, which contained S57R and K65R mutations respectively (Table 3B). No variants with G57 at this position were identified. Although both scFvs incorporated mutations that were predicted by the structural analysis, no variants with mutations at position 236 were obtained.

3.3. Combinatorial mutations and evaluation of the recognition of the new scFvs by SPR

A new set of scFv variants, which contained a combination of mutations that were present in the scFvs isolated from directed evolution and semi rational approaches were constructed. These variants and the ones that were already selected by maturation processes (Table 3B) were expressed, purified and evaluated to compare their binding capacity to Css2 toxin by means of SPR. One of these variants named scFv ER-5 with the combination of R57, R65, N110 and G208 (Table 3B and Fig. 2) showed a suitable binding capacity to Css2 toxin, with an affinity constant (K_D) of 10 nM (Table 4). Furthermore, Css4 toxin which is another component in *C. suffusus* venom (S Cestèle et al., 1998; Espino-Solis et al., 2011) was bound by scFv ER-5 with an affinity of 2.3 nM. It is worth mentioning that the (K_D) of scFv ER-5 for the Css4 toxin was higher than the corresponding value for the Css2 toxin. The difference in these affinities is explained by the fact that the association rate constant (k_{on}) for Css4 is slightly higher than that for Css2, while the dissociation rate constant (k_{off}) is similar for both toxins (Table 4).

Characteristically, variants derived from scFv C1 exhibited high cross reactivity against various toxins. scFv ER-5 besides recognizing Css2 and Css4 toxins, exhibited an improved binding to Cn2 and Cll1 toxins (Table 4). scFv ER-1 which showed the best affinity against Cll2 and Ct1a toxins also maintained an equally good binding to Cn2 and Cll1 toxins (Table 4). Complementarily, scFv ER-6 that contains only three changes with respect to scFv RU1, increased its affinity (K_D) to the Cn2 toxin 5-fold (Table 4).

The combination of aforementioned mutations, improved the binding of this scFv with those toxins as a consequence of a slower dissociation rate (*k*off) during the molecular interactions. This slower dissociation rate is reflected as an increase in the interaction time (T_R; time of residence, see Material and Methods section) (Table 4).

Table 4
Determination by SPR of the affinity of several variants from the C1 family for the different toxins.

Determination of the kinetic constants				T_R (min)
scFv	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (M)	
Css2 toxin				
ER-5[a]	$1.2 \times 10^5 \pm 0.2$	$1.2 \times 10^{-3} \pm 0.05$	$1 \times 10^{-8} \pm 0.008$	14
Css4 toxin				
ER-5	4.8×10^5	1.1×10^{-3}	2.3×10^{-9}	15
Cn2 toxin				
RU1	6.8×10^4	4.0×10^{-4}	5.9×10^{-9}	42
ER-1	2.1×10^5	2.1×10^{-4}	1.0×10^{-9}	79
ER-5[b]	$2.1 \times 10^5 \pm 0.7$	$2.7 \times 10^{-4} \pm 0.3$	$1.2 \times 10^{-9} \pm 0.5$	62
ER-6[b]	$1.5 \times 10^5 \pm 0.3$	$1.7 \times 10^{-4} \pm 0.4$	$1.1 \times 10^{-9} \pm 0.4$	98
Cll1 toxin				
RU1	13.2×10^4	9.2×10^{-4}	6.8×10^{-9}	18
ER-1[b]	$3.8 \times 10^5 \pm 1$	$5.0 \times 10^{-4} \pm 0.7$	$1.3 \times 10^{-9} \pm 0.3$	33
ER-5[b]	$2.2 \times 10^5 \pm 0.7$	$4.7 \times 10^{-4} \pm 1$	$2.1 \times 10^{-9} \pm 0.5$	35
ER-6	1.5×10^5	8.8×10^{-4}	2.5×10^{-9}	19
Cll2 toxin				
RJI-1	3.7×10^5	2.7×10^{-3}	7.2×10^{-9}	6.2
ER-1[b]	$4.7 \times 10^5 \pm 0.8$	$2.7 \times 10^{-3} \pm 0.3$	$5.8 \times 10^{-9} \pm 0.3$	6.2
Ct1a toxin				
RJI-1	1.9×10^5	3.2×10^{-3}	1.7×10^{-8}	5.2
ER-1[b]	$2.7 \times 10^5 \pm 0.02$	$2.9 \times 10^{-3} \pm 0.6$	$2.9 \times 10^{-8} \pm 0.1$	5.7

Kinetic constants were determined in individual assays, with the exception of scFv ER-5.
[a] n = 3.
[b] n = 2. Time of residence (T_R) of the scFvs with each toxin were calculated based on the k_{off} values and are expressed in minutes.

3.4. Neutralization tests against the Css2 toxin

The ability of scFv ER-5 to neutralize Css2 toxin was evaluated in CD1 mice by intraperitoneal injections. scFv ER-5 was mixed with 1 or 2 LD_{50} of toxin in different molar ratios. The results of these tests are shown in Table 5. As it can be seen, with a molar ratio of 1:10, scFv ER-5 was able to neutralize both LD_{50} of the Css2 toxin without observable envenoming symptoms. Similar effects were observed when lower molar ratios, such as 1:5 (toxin:scFv) were injected, indicating an acceptable neutralizing capacity of scFv ER-5. Neutralization potency was contrasted when 1 LD_{50} was injected into control group where only 2 out of 10 mice survived. These results indicated that the toxicity of the Css2 toxin had been underestimated. Furthermore, the neutralizing capacity of ER-5 could be also valued when 2 LD_{50} were injected. No survivors left in the control group while all the mice in the experimental one were protected from envenomation even using a molar ratio of 1:5. It is noteworthy that the amount of Css2 toxin present in $2LD_{50}$ used in this assay (1.5 μg), is equivalent to that contained in $6LD_{50}$ of whole venom (Estrada et al., 2007 and Table 2), given an abundance of 2.8% (Martin et al., 1987).

3.5. Neutralization tests against Cn2 and Cll1 toxins

The ability of the scFvs ER-1, ER-5 and ER-6 to neutralize Cn2 and Cll1 toxins was determined in *in vivo* experiments (Table 6). All of the tested scFvs exhibited a good neutralizing capacity against

Table 5
Ability of scFv ER-5 to neutralize Css2 toxin.

scFv	LD_{50}	Molar ratio (toxin:scFv)	Survivors (alive/total)
ER-5	1	1:10	10/10
ER-5	1	1:5	10/10
ER-5	2	1:10	10/10
ER-5	2	1:5	10/10
Toxin alone (control)	1	1:0	2/10[a]
Toxin alone (control)	2	1:0	0/10[a]

[a] Symptoms of envenoming were severe.

Table 6
A. Neutralizing capacity against 2 LD_{50} of the Cn2 toxin. B. Neutralizing capacity against one LD_{50} of the Cll1 toxin.

A.			
scFv	Molar ratio (toxin:scFv) survivors (alive/total)		
	1:5	1:2	1:1
ER-5	10/10	ND	ND
ER-6	10/10	10/10	8/8[a]
ER-1	ND	10/10	4/10[b–c]
Toxin alone (Control)	1/10[c]		
B.			
scFv	Molar ratio (toxin:scFv) Survivors (alive/total)		
	1:10	1:2	1:1
ER-5	10/10	8/8	8/8[a]
ER-6	ND	10/10[a]	ND
ER-1	ND	10/10	10/10[a]
Toxin alone (Control)	5/10[c]		

The presence and intensity of the symptoms of intoxication are categorized as follows: a; mild, b; moderate, c; severe. ND: Not determined.

these toxins. It is important to comment that although Cn2 toxin is approximately 7 times more toxic than Cll1 toxin, all the generated variants were able to neutralize 2 LD_{50} of that toxin. However, some differences in the neutralizing capacity were observed when lower molar ratios (toxin:scFv) were injected. scFv ER-6 was able to neutralizing 2 LD_{50} of Cn2 toxin even at a molar ratio 1:1. Similarly, scFvs ER-1 and ER-5 neutralized Cll1 toxin at this lowest molar ratio. In both cases only mild intoxication symptoms were observed. There are few reported the scFvs or nanobodies with this neutralization potency against several scorpion toxins (Abderrazek et al., 2009; Rodríguez-Rodríguez et al., 2012).

3.6. Neutralization capacity of scFv ER-5 when challenged with whole venom of C. suffusus

Because ER-5 variant neutralizes Css2 toxin, its ability to neutralize the whole venom of *C. suffusus* was evaluated. The venom used in this study was obtained from animals that were recently collected and milked the same day of the experiments to guarantee the highest toxicity. Two or three LD_{50} of venom were mixed with scFv ER-5 alone or in combination with scFv LR and pre-incubated before being administered to mice (Table 7). The scFv ER-5 alone was able to neutralize 2 LD_{50} of *C. suffusus* venom without the appearance of envenoming symptoms. These stimulating results prompted us to challenge ER-5 variant using 3 LD_{50} of whole venom. In this case, 9 out of 10 mice survived showing some envenoming symptoms even when higher molar ratios of toxin:antibody (1:30) were used (Table 7). To achieve complete neutralization of the venom, a second assay was performed using a combination of variants ER-5 and LR (which binds to a second non-overlapping epitope that is present in the Css2 toxin). This mixing was capable of neutralizing 3 LD_{50} of whole venom even with a lower molar ratio such as 1:5 of each scFv, revealing a complementary effect. Additionally, competition assays by means of SPR, confirmed that scFv ER-5 recognizes a different epitope than the one recognized by scFv LR (Fig. 4) and competes for the site recognized by scFv RU1. These results confirmed that both scFvs simultaneously bind to Css2 toxin on the already identified epitopes (Riaño-Umbarila et al., 2016).

4. Discussion

In previous studies, the parental scFvs 3F and C1 were evolved using *in vitro* affinity maturation strategies to obtain a group of scFvs that were capable of neutralizing some scorpion toxins (Riaño-Umbarila et al., 2016, 2013, 2011, 2005). During these processes, we found that the scFvs, in addition to recognizing their original target (the Cn2 toxin) were able to recognize other toxins. In particular, the scFvs derived from the parental scFv C1 showed a greater cross reactivity than the scFvs derived from scFv 3F when evaluated by ELISA assays (data not shown). When applied to the improvement of C1 family of variants, directed evolution was not as straight as in the case of parental scFv 3F (Riaño-Umbarila et al., 2011, 2005) and other reported studies (Barbas et al., 1994; Boder et al., 2000; Deng et al., 1994). The C1 variants that were obtained using this process did not lead to significant improvements in their recognition to the toxins after several cycles of directed evolution. The combination of the detected changes into a single variant (scFv RU1) (Table 3A) resulted in broader recognition properties. This feature is reflected as an increase in its capacity to recognize various scorpion beta-neurotoxins. The variants derived from scFv RU1 (second offspring from C1, see Fig. 2), RJI-1, RJI-2 and RJI-3, exhibited improved binding to different toxins (Fig. 3A-B). Interestingly, the SPR analysis showed that specific residues at position 110 may influence the improved recognition towards certain toxins. The scFv RJI-1 containing T110 (Table 3A) showed better binding to the Cll1, Cll2 and Ct1a toxins (Fig. 3B), whereas RJI-2 containing N110 (Table 3A) exhibited improved binding to Css2 (Fig. 3A) and Cn2. This behavior is explained because the amino acid 110 is located in the V_H-CDR3, which is one the main elements involved in antigen recognition (Sundberg, 2009; Xu and Davis, 2000).

As already mentioned after the different key mutations were combined, significant improvements in the binding properties were observed in this scFv family. We decided to perform a

Table 7
Neutralization tests using whole venom.

A. Pre-incubation			
scFv	LD_{50}	Molar ratio (toxin Css2:scFv)	Survivors (alive/total)
ER-5	2	1:10	10/10
ER-5	2	1:20	10/10
ER-5	3	1:30	9/10[a–b]
ER-5 and LR	2	1:10* (1:20)	10/10
ER-5 and LR	3	1:10* (1:20)	10/10
ER-5 and LR	3	1:5*(1:10)	10/10
Venom alone (Control)	2	1:0	0/10[c]
Venom alone (Control)	3	1:0	0/10[c]

The presence and intensity of the symptoms of intoxication are categorized as follows: a; mild, b; moderate, c; severe. The molar ratios were calculated in relation to the proportion of Css2 toxin within the venom (2.8%). *These molar ratios refer to each scFv.

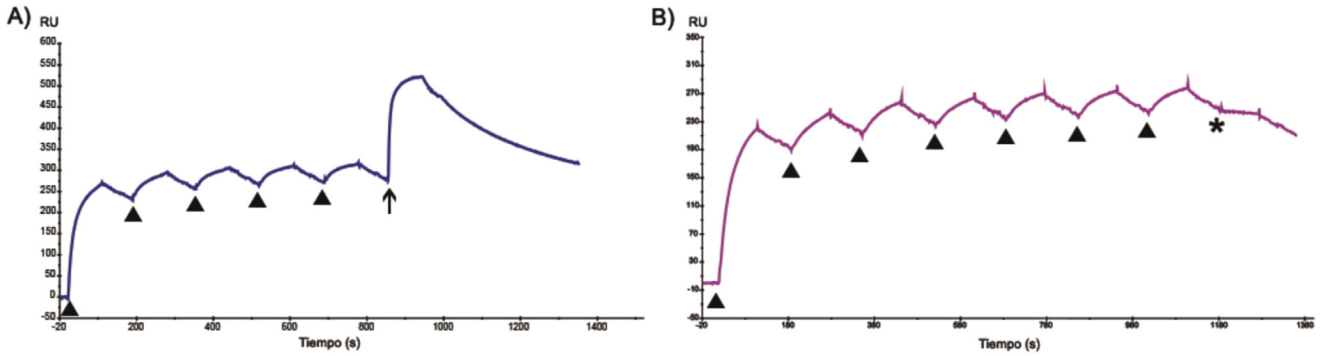

Fig. 4. Competitive binding of scFvs ER-5 and LR to Css2 toxin. Sensograms of epitope competition. A) Triangles indicate injections of scFv ER-5 (1 mM) to achieve saturation (5 injections) and the arrow indicates injection of scFv LR (0.5 mM). B) Triangles indicate injections of scFv ER-5 (1 mM) to achieve saturation (5 injections) and the asterisk indicates injection of scFv RU1 (1 mM).

structural analysis to identify the residues that could establish better interactions with their targets, particularly the interactions between RJI-2 and the Css2 toxin (Fig. 3A). As described in the results section, positions 57, 65 and 236 were mutagenized and combined to generate multiple mutants.

Two scFvs (ER-3 and ER-4) were obtained through *in vitro* maturation using the semi-rational approach (Fig. 2 and Table 3B). Both variants exhibited a good binding to the Css2 toxin, but we had expected that the obtained scFvs should have shown even better affinity in accordance to successful reports where this approach was used (Barderas et al., 2008; Kiyoshi et al., 2014). Although no significant improvements were obtained, scFv ER-3 it is worth noting that this variant it was also selected during the process of directed evolution. Therefore, the mutation present in this variant (R57) might be relevant depending on the sequence context.

Furthermore, the scFv ER-1 obtained from directed evolution maturation processes contained a single change (D208G) located in (FW3-V_L) (Table 3B and Fig. 2). During exploratory assays, it exhibited improved binding capacity toward Css2, Ct1a (Fig. 3C and D) and other toxins (Cn2 and Cll1) (Table 4). To understand the contribution of this mutation to the improvement of binding, the amino acid sequence of scFv ER-1 was compared with its corresponding germline sequences available in the V-BASE2 database (Retter et al., 2005). We found that the G208 residue is highly conserved in the FW-3 of the Vλ families. It is currently known that reverting mutations to the germline consensus sequences can positively influence the stability of antibodies, proteins and enzymes (Amin et al., 2004; Chromikova et al., 2015; Lehmann et al., 2000; Wirtz and Steipe, 1999).

Moreover, when scFv ER-1 was characterized, we demonstrated that this scFv exhibited a broadened binding capacity towards Cll1, Cll2 and Ct1a toxins (Table 4). This is the first time that the binding of Ct1a by an antibody fragment has been evaluated and it constitutes a promising result because Ct1a is the major toxic component of *C. tecomanus* scorpion venom. We could improve the binding properties of scFv ER-1 to neutralize the whole venom of the scorpions *C. limpidus* and *C. tecomanus*.

4.1. Combining mutations

Several scFvs were generated by combining mutations that occurred during these processes of *in vitro* affinity maturation. As has been suggested, the effect of specific mutations on the functional properties of a protein can be different depending on the sequence context (interaction of a certain residue with different partners). We found that the combination of the mutations present in scFv ER-5 (Table 3B and Fig. 2) resulted in a good affinity to Css2 toxin. This scFv, with a K_D of 1×10^{-8} M (Table 4), showed the ability to neutralize 2 LD_{50} of both Css2 toxin and the whole venom of *C. suffusus* using molar ratios of 1:5 and 1:10 (toxin: scFv), respectively. No envenoming symptoms were observed up to 48 h (Tables 5 and 7). Furthermore, it was demonstrated that mixing scFvs ER-5 and LR resulted in significant levels of protection reaching complete neutralization of 3 LD_{50} of whole venom even with a molar ratio of 1:5 (Table 7). Similar results using LR and RU1 had been reported with the venom of *C. noxius* (Riaño-Umbarila et al., 2016). In summary, the neutralization of the main toxins with two antibodies that bind to two different epitopes (Fig. 4) would guarantee the complete neutralization of the venoms. Based on these results, we believe that scFv ER-5 is a candidate that could be part of a recombinant antivenom of human origin against stings from venomous Mexican scorpions. Furthermore, scFv ER-5 exhibited a good neutralizing capacity against Cll1 and Cn2 toxins (Table 6). Nevertheless, we will continue generating optimized scFvs with a broader binding and neutralizing properties.

Another variant generated in this work was scFv ER-6 (Table 3B and Fig. 2), which proved to be a variant with a good neutralizing capacity against Cn2 and Cll1 toxins. Its affinity parameters allowed us to observe that differences in K_D constants were minimal (Table 4). In terms of the variability among dissociation constants (k_{off}), we have noticed that for a determined toxin, scFvs with higher retention times ($T_R = 1/k_{off}$) showed a better neutralizing capacity (Riaño-Umbarila et al., 2016). Variants ER-1 and ER-5 with T_Rs of approximately 30 min, neutralized Cll1 toxin at molar ratios as low as 1:1 (Table 6) reaching 100% survival rates among challenged mice. Furthermore, scFv ER-6 which showed T_Rs of approximately 90 min protected mice against the most potent toxin (Cn2) at a molar ratio 1:1. These results indicate that longer times of residence are necessary to neutralize more potent toxins. Similar inferences can be raised for variants obtained from scFv 3F which bind to the other epitope (Riaño-Umbarila et al., 2016, 2011; Rodríguez-Rodríguez et al., 2012). However, lower binding strengths (higher K_D values) are necessary to neutralize the epitope recognized by scFv RU1 variants when compared to LR variants.

The analyses indicate that the selected mutations and their combination resulted in an improvement of the inhibitor antibodies because they are now able to recognize not only Cn2, Cll1 and Css2 toxins but also Ct1a and Cll2 toxins. This is worth noting since these mutant scFvs are the product of maturation processes (directed evolution and semi-rational approaches) that were only conducted against Css2 toxin. The variants exhibits a broader specificity, which allows them to recognize several toxins from the venom of scorpions of the genus *Centruroides* with similar efficiency (Table 4). Although this property could be explained by the

high sequence identity shared among scorpion toxins, contrasts with the fine specificity of the toxins on different sodium channel subtypes (Schiavon et al., 2006).

Binary models were constructed with different toxins to understand how a few mutations increased the cross reactivity of this family of scFvs (see materials and methods). The analyses were focused on the toxin area that was recognized by scFv RU1, which involved 14 amino acid residues of Cn2 toxin (indicated by triangles in Fig. 1). Among the various toxins present in the venoms of *Centruroides* scorpions, many of these residues are conserved or in some cases are similar. The analyses of the electrostatic potential of the toxins confirmed their similarity in the distribution of negative and positive potentials compared to Cn2 toxin (see Fig. 5A and B). Consequently, due to the high identity among toxin sequences, the high cross reactivity of scFv ER-1 is explained by the complementarity of the positive and negative potentials of the respective areas on the CDRs V_H 2 and 3 (Fig. 5A and B). It was observed that the mutations that were incorporated into scFvs that exhibited improved binding to different scorpion toxins corresponded to positions 57 (S or R) and 110 (N or T) (Table 3A). These mutations are located at the CDR2-V_H and CDR3-V_H respectively, and did not modify the positive and negative electrostatic potentials (see Fig. 5A and B). We evaluated the binding of scFv ER-1 (S57, T110, I164 and G208) to the Cn2, Css2, Cll1, Cll2 and Ct1a toxins using *in silico* structural models of the binary complexes. In particular, the N57S mutation resulted in a decrease of the size of the corresponding side chain which favored an adequate fitting of the CDR2-V_H region with each of the different toxins, giving rise to a more suitable surface complementarity. Similar effects were observed within the crystal structure of scFv RU1-Cn2 at positions 54 and 56, where the presence of the smallest side chains corresponding to Gly residues, might confer some flexibility to this protein area in scFvs which would propitiate a better binding to different toxins (Riaño-Umbarila et al., 2016). In that structural model, residue H110 forms a hydrogen bond with the oxygen of the carbonyl carbon of residue D108, likely favoring the stabilization of the CDR3-V_H which harbors these residues. Furthermore, we observed that the presence of T at position 110 increases the distance between these two residues. In scFv RU1, distance is 2.95 Å, whereas in scFv ER-1 it is 3.65 Å (Fig. 5B and C) indicating a loss of interaction between residues 110 and 108. The lack of this contact, might favor the flexibility associated to CDR3-V_H resulting in a broadening of the capacity of binding to various toxins. This flexibility would maintain the cross reactivity without losing affinity strength for the different toxins (Table 4). This property of the variants here reported, might represent a nice example of the induced fit phenomenon, which has been shown to be closely related to the flexibility of CDR3-V_H (Bosshard, 2001; Rini et al., 1992; Stanfield et al., 2007). However, it is necessary to determine the three dimensional structure of the scFv alone and in complex with toxins to confirm this hypothesis. We must also explore different changes at positions 57 and 110 to diminish the

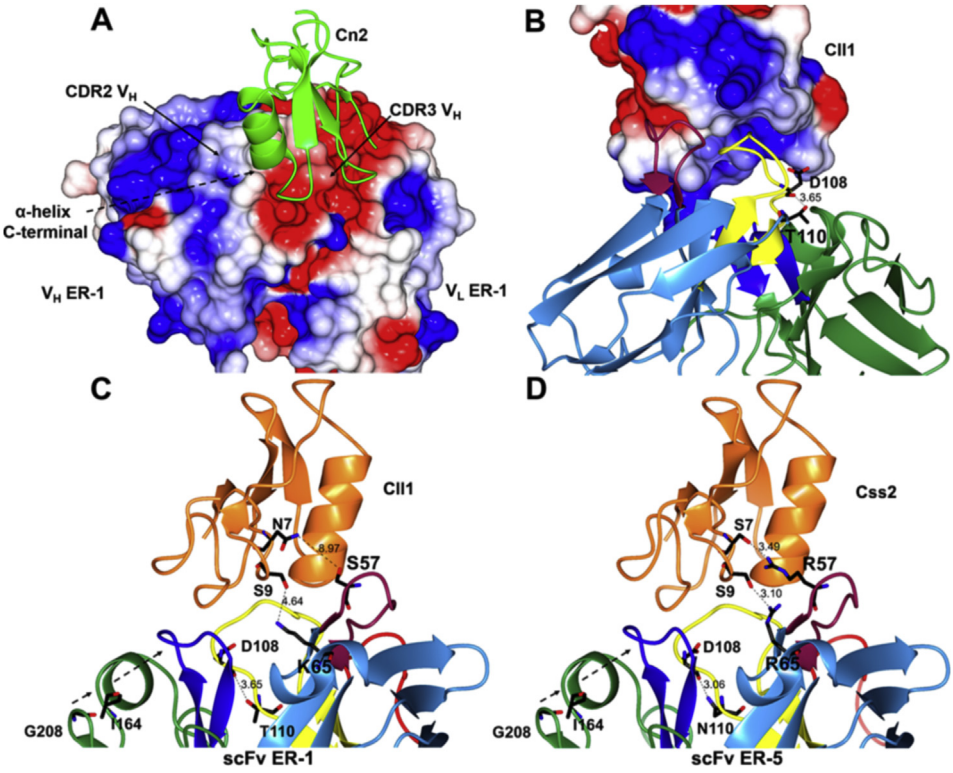

Fig. 5. Models of the binary scFv-toxin complexes. A) Model of scFv ER-1-Cn2 complex. ER-1 is colored according to the electrostatic potential. Negatively and positively charged surfaces are colored red and blue, respectively, while neutral surface is colored white. Note that positively charged CDR2-V_H and negatively charged CDR3-V_H interact with the α-helix in the C-terminal region of Cn2 toxin (green). B) Model of scFv ER-1-Cll1 complex. Cll1 toxin is colored according to the electrostatic potential, while scFv ER-1 V_H is colored light blue and the ER-1 V_L is shown in green. Notice that the long CDR3-V_H (yellow) and CDR2-V_H (dark purple) interact with regions of complementary charge in Cll1 toxin. The distance between T110 and the carbonyl oxygen group of residue D108 is indicated (3.65). C) Representation of the interactions between scFv ER-1 (same color codes as in panel B) and Cll1 toxin (orange). Distances among residues S 57 and K65 of scFv ER-1 with the respective amino acids at positions N7 and S9 of Cll1 toxin are indicated, to be compared with the same positions of scFv ER-5 and Css2 toxin shown in panel D. D) Model of scFv ER-5-Css2 complex. Notice that compared to panel C, the distance between residue N110 and carbonyl oxygen group of residue D108 is lower (3.06). Additionally, in contrast with (C), due to proper distances between residues R65 of FW3 and R57 of CDR2 of V_H with residues S7 and S9 of the Css2 toxin, corresponding hydrogen bonds are formed. Location of mutations G208 and I164 are also shown. Details regarding this figure are described in the text. Distances are indicated in Angstroms (Å).

supposed "stiffness" at CDR2-V_H and CDR3-V_H, thus generating more flexible loops hopefully increasing scFv cross reactivity.

Conversely, when scFvs contain residue N110, the distance between this amino acid and the oxygen in the carbonyl group of residue D108 is reduced to 3.06 Å (Fig. 5D) approaching the value observed in scFv RU1 (2.95 Å). These observations suggest that CDR3-V_H might be stabilized through N 110, which in turn would be determining a decrease in the flexibility and consequently narrowing the cross reactivity potential of the variants that contain this change. This would explain why scFvs ER-5 and ER-6 preferentially neutralize Cn2 and Css2 toxins, which share a greater similarity (≈90%) and therefore a greater complementarity with the corresponding surfaces on these scFvs.

In the model in which scFv ER-5 binds to Css2, we observed that the side chains of residues R57 and R65 are longer than N57 and K65, favoring the formation of hydrogen bonds with toxin residues S7 and S9, respectively (Fig. 5C and D). These new interactions might have been influential to reach an adequate retention time which was sufficient to neutralize Css2 toxin (Tables 4 and 5).

Other potentially important changes identified were S164I and D208G. The models did not help to address a logical interpretation of these results because these residues do not interact with the toxins (Fig. 5C and D). However, the experimental results in terms of affinity and neutralizing capacity, showed that these changes play an important role in improving the binding of scFv ER-1 with the toxins compared with scFv RJI-1 which does not contain D208G mutation and exhibited a lower association rate (Table 4). These observations show that mutation D208G despite being located outside the contact areas with the toxin, it might be beneficial indirectly reflected as an increase in the association rate during the kinetics of scFv-toxin interaction.

We speculate that D208G mutation, which reverted to a germline residue and considering its side chain minimal size from the structural point of view, it would increase the flexibility of the loop that connects two β strands including the loop that contains the S164I mutation (Fig. 5C and D, dashed arrow). In summary, this mutation would be associated to a stabilizing effect on the binding conformation of scFvs, reflected as an increase of association rate. Considering these speculations, it would be necessary to evaluate the effects on the optimization of a binding conformation by incorporating some Gly residues to determine if the supposed increase of flexibility in this area, results in a broader cross reactivity.

5. Conclusion

The results reported here represent an advance in the generation of antivenoms with less complexity and higher cross reactivity against the toxins of primary medical importance and their corresponding venoms from Mexican venomous scorpions. We focused on the most abundant and toxic (lethal) components of the venoms in accordance with the criteria recently suggested to consider a toxin as medically relevant (Laustsen et al., 2015) These new scFvs have the ability to neutralize several toxins by incorporating only a few changes at the key positions identified in this work. Although we know that a single antibody fragment can neutralize the effects of some venoms (*C. noxius* and *C. suffusus*), we have demonstrated that two neutralizing antibodies that recognize different epitopes on the toxins can neutralize toxic effects more efficiently and safely when they are combined (this work and Riaño-Umbarila et al., 2016). We believe that the generation of new scFvs derived from members of the family of toxin inhibitors here reported will allow to neutralize a greater number of toxins and scorpion venoms. When added to the already developed scFvs, the mix will constitute a polyvalent antivenom that might replace the current equine product.

Ethical statement

The authors of this manuscript declare that they all have followed the ethical requirements for this communication. The experiments in which animals alive were used and reported in this manuscript were approved by Ethical Committee of our Institute of Biotechnology from the National Autonomous University of Mexico.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Research Article

A Study of DNA Damage in Buccal Cells of Consumers of Well- and/or Tap-Water Using the Comet Assay: Assessment of Occupational Exposure to genotoxicants

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Because of concerns that natural aquifers in the region of Todos Santos (Baja California Sur, Mexico) might be contaminated by organochlorine pesticides and heavy metals, a case–control study was conducted among consumers and non-consumers of well- and/or tap-water to determine risks to human health. This study was based on a genotoxic evaluation of buccal cells using the Comet assay technique. Levels of DNA damage in the consumers group were significantly higher than those of the control group. However, occupational exposure to genotoxicants showed to be the critical factor rather than water consumption. Taking into account the professions of well- and/or tap-water consumers, agricultural workers exposed directly (those who fumigated) or indirectly (those not involved in fumigating) to agrochemicals showed greater genetic damage than controls. This difference persisted even when age, and whether the person smoked or consumed alcoholic drinks were considered. These factors were not associated with the level of genetic damage observed. Chemical analyses of organochlorine pesticides and heavy metals were carried out to evaluate the water quality of wells, faucets, and surface water of canals consumed by the population and/or used for irrigation. High concentrations of α and β endosulfan were detected in water of surface canals. Although our inventory of agrochemicals employed in the region showed the use of products considered carcinogenic and/or mutagenic, they were not detected by the analytical techniques used. Heavy metals (arsenic, mercury, and lead) were detected in water of some wells used for irrigation and human consumption. Environ. Mol. Mutagen. 58:619–627, 2017. © 2017 Wiley Periodicals, Inc.

Key words: genotoxicity; water consumption; public health; pesticides; heavy metals

INTRODUCTION

One of the most serious problems that human and wild animal populations confront is the damage caused by exposure to residues of harmful substances of anthropogenic or natural origin that may be released into the environment. Ingestion of contaminated food and water causes the transfer and magnification of such residues through the trophic chain in a phenomenon that has been described on many occasions [see Borgå et al., 2004, among others]. Pesticides are one of the contaminants to which people are most often exposed due to their utilization at home, in industries, for maintenance of green areas and bodies of water, cattle-raising, etc. It is well known that chronic exposure to pesticides affects many biological functions and processes. They can induce alterations of the reproductive [Upson et al., 2013; Chiu et al., 2015], immune [Dewailly et al., 2000], and nervous systems [Viel et al., 2015], while also causing diseases such as Parkinson's [Rugbjerg et al., 2011], Alzheimer's [Richardson et al., 2014], diabetes [Del Razo et al., 2011], and certain types of cancer [Alavanja et al., 2004; ATSDR, 2007; Provost et al., 2007]. Cohn et al. [2015], for example, demonstrated the relation between breast cancer and exposure in utero to agricultural chemicals before birth.

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Epigenetic studies have also proven that the alterations induced by agricultural chemicals can be transmitted to as many as three succeeding generations [Manikkam et al., 2014].

A previous monitoring carried out by our team detected the presence of the pesticide lindane in wild crustaceans collected in water canals that traverse the village of Todos Santos, Baja California Sur, Mexico. These waters are used in some homes for personal hygiene and/or to water gardens, or are provided to animals such as cattle, sheep, goat, and pig. These canals are fed by water from the Todos Santos aquifer, so there is a possibility that this natural deposit is contaminated with agricultural chemicals. On the other hand, monitoring conducted in wells of the region has detected arsenic produced by processes of geological erosion and the extensive mining activity that took place in this zone in earlier periods [Colín-Torres et al., 2014]. Arsenic can cause harmful effects in the skin [Meek and Hughes, 1992], and the neurological and reproductive systems [Buchet and Lison, 2000]. It can also cause epigenetic alterations that modify gene expression [Fragou et al., 2011].

The water consumed by the population in the study area is extracted by pumps, but receives no treatment or control to ensure its quality. For generations, these hard waters have been habitually ingested in varying frequency by a large part of the human population of Todos Santos.

Determining the active substances contained in the chemicals of natural and anthropogenic origins that are disseminated through different environmental matrixes is an indispensable step in evaluating risks for human health due to chronic exposure and/or ingestion. With respect to pesticides, there are analytical techniques that are capable of identifying some of those active substances; however, their use is complicated by high costs and the wide range of formulations contained in pesticides, many of which remain unknown. The use of biomarkers allows the application of the molecular epidemiology approach to the detection of early effects in exposed populations. This, in turn, facilitates the implementation of preventive methods designed to offset the harmful effects by considering the genetic characteristics of populations. Several markers are now available to monitor exposure of humans to environmental mutagens and carcinogens. The study of genotoxic effects caused by xenobiotics allows the identification of hazards in environmental risk assessment [Valverde and Rojas, 2009]. Epithelial cells—specialized in many organs—have the potential to serve as biomatrixes for evaluating genotoxicity and, therefore, also as biomarkers of genotoxic effects [Rojas et al., 2014].

To our knowledge, no studies have yet been conducted to assess the genotoxic potential of consuming hard water from wells and/or taps, which could represent a risk for the health of the inhabitants of Todos Santos, Baja California Sur, Mexico. Given the possibility that nearby natural aquifers may be contaminated with agrochemical products and heavy metals, and that consumers' health could be affected, we conducted a genotoxic evaluation using the case–control method and two population groups, as follows: (a) consumers of well- and/or tap-water; and (b) a reference group of people who did not consume water from those sources. Parallel to this, chemical analyses of the waters were performed to detect the possible presence of organochlorine pesticides and heavy metals.

MATERIALS AND METHODS

The Study Area

The study area is located between coordinates 23°26'21.87" and 23°31'36.36"N-110°10'39.10" and 110°17'27.33"W, in the extreme southwestern zone of the Baja California Peninsula, Todos Santos, Baja California Sur, Mexico, between the Pacific Ocean and the Sierra de la Laguna mountains.

Cytogenetic Biomonitoring

Selection of Individuals

Buccal cell donors were randomly-selected from natives and current residents of the village of Todos Santos or of neighboring farms. A total of 107 males who had drunk well- and/or tap-water always or occasionally throughout their lifetimes were selected for the case group; while 18 women and 22 men from other regions who were not natives of Todos Santos and had consumed only bottled water, formed the control group. Subjects in both groups were classified according to age as $\leq$40 versus $>$40 years old.

Because the main source of employment in the study region is agriculture, and the handling of agrochemicals is associated with increased genetic damage, the consumers of well- and/or tap-water were categorized according to their occupation: (1) conventional agriculture workers (fumigators and non-fumigators); (2) organic agriculture workers (no use of agrochemicals); and (3) workers not associated with agriculture (businessmen, civil servants, storekeepers, etc.). No individuals in the control group had occupational contact with agrochemicals.

For each donor we gathered information on: (a) demographics (age, place of residence); (b) medical history (diseases, family antecedents of cancer); (c) previous and current occupation; (d) years of occupational exposure to chemicals; and (e) lifestyle habits (diet, smoking, alcohol consumption) (Tables I and II). All donors were informed clearly and honestly of the study's objective and their written consent was obtained.

Sampling of Buccal Cells

Sampling of buccal cells was carried out during the growing season for agricultural crops according to Rojas et al. [1996]. Buccal cell samples were obtained from each individual by rubbing the inside of their cheeks with a sterile cotton swab and collecting the cells in a microcentrifuge tube containing 400 µl of deionized water. All samples were refrigerated for transportation to the laboratory (max. 72 h) and then centrifuged at 6,000 rpm for 3 min before being rinsed with deionized water. This procedure was performed three times for each sample. Before beginning each analysis, the appropriate volume of cell suspension for single-cell gel electrophoresis (SCGE) was estimated by staining cells with trypan blue and counting the viable cells in a Neubauer chamber.

Genotoxicity in Buccal Cells

Genotoxicity in buccal cells was evaluated by SCGE, a technique that reveals evidence of single and double strand breaks in DNA and detects transient DNA breaks present as intermediates in the process of

TABLE I. Age, Occupation, and Lifestyle Habits of Buccal Donors, Categorized According to Their Consumption of Water and Handling of Pesticides

Consumers	Purified water control group		Well/tap-water exposed of pesticides		Well/tap-water no exposed of pesticides	
Age groups (yr)	≤40	>40	≤40	>40	≤40	>40
Age (yr)[a]	32 ± 5	53 ± 9	30 ± 6	54 ± 9	26 ± 7	49 ± 3
Total no. individuals	20	20	46	35	16	10
Occupation (no.)						
Conventional agriculture workers	—	—	46	35	—	—
Organic agriculture workers	—	—	—	—	8	5
Workers not associated with agriculture	20	20	—	—	8	5
Lifestyle habits (%)						
Smokers	45	24	59	36	44	22
Drinkers	55	48	39	52	31	44
Consumption of well-and/or tap-water (%)						
Never	100	100	—	—	—	—
Occasionally	—	—	48	52	75	44
Always	—	—	52	48	25	56

[a]Results are displayed as mean ± standard deviation.

TABLE II. Pesticide Exposure in Conventional Agricultural Workers

	Pesticide handlers	No pesticide handlers
Conventional agriculture workers (no.)	57	24
Years working in agriculture (%)		
≤10	18	19
10–20	14	5
>20	25	—

DNA repair. Microscope slides were pre-coated with a 0.5% standard agarose layer (Sigma). A second layer containing 5×10^4 to 10×10^4 cells in 0.5% low-melting point (LMP) agarose (Sigma) was added to each pre-coated slide, followed by a final layer of 0.5% LMP [Rojas et al., 1996]. Slides were incubated for 1 h in a lysis solution (NaCl 2.5 M; EDTA trisodium salt trihydrate 100 mM; Tris base 10 mM, DMSO 10%, Triton X-100 1%, pH 10) at 4°C. DNA unwinding was performed in an electrophoresis solution (NaOH 0.3 M, EDTA trisodium salt trihydrate 0.85 mM, pH 10) for 15 min. After this step, horizontal electrophoresis was performed at 4°C for 20 min using the same electrophoresis solution (at 0.75 V/cm, 300 mA). After electrophoresis, the slides were rinsed three times for 5 min each with a neutralizing solution (Tris base 400 mM, pH 7.5) and then three times for 5 min each with ice-cold absolute ethanol. All steps after lysis were performed under yellow lighting. Cells were analyzed by fluorescence microscopy (400×) with an excitation/emission filter (535–550/580) using a 25 μL/mL ethidium bromide solution. Tice et al. [2000] and Collins [2004] recommended evaluating 100 cells per organism (50 cells per slide; two replicates), but in this study, we evaluated 25 cells per slide with four replicates per donor. Bigaud Guerin [2008] validated this technique statistically in our laboratory. The evaluation of genetic damage was conducted by visual inspection of the slide. The 25 cells on each slide were grouped into five categories according to tail intensity (DNA). A value between 0 and 4 was given for each comet class: (0) = undamaged; (1) = 1–20%; (2) = 21–40%; (3) = 41–60%; and (4) = 61–80%, of DNA in the tails. The total amount of DNA strand breakage was expressed as arbitrary units (AU) [Collins et al., 1997] and calculated using the following equation: $N\,0 \times 0 + N\,1 \times 1 + N\,2 \times 2 + N\,3 \times 3 + N\,4 \times 4$, where N is the number of cells scored in each category [Collins, 2004].

Analysis of Contaminants in Water for Human and Animal Consumption

Analyses for organochlorine pesticides and heavy metals were conducted in water from 19 wells, tap-water from six dwellings, and four samples of surface water from irrigation canals. Tests using gas chromatography—analytical method EPA 8081 A 1996—were performed for the following pesticides: aldrin, DDT, DDD, DDE, chlordane, α, β-BCH dieldrin, α and β-endosulfan endrin, endrin ketone, γ-BCH, heptachlor, epoxide heptachlor, hexachlorobenzene, methoxychlor, toxaphene, δ BHC, endrin aldehyde, endosulfan sulfate, alachlor, atrazine, cyanazine, deltamethrin, metolachlor, mirex, pendimethalin, terbuthylazine, trifluralin, and simazine. The presence of four heavy metals was determined by atomic absorption spectrophotometry for mercury (NMX AA-051-SCFI-2001/EPA7470A-1994), and arsenic, lead, and cadmium (NMX AA-051-SCFI-2001/EPA6010C-2007). All analyses were performed individually for each sampling site at the Laboratorio ABC Química Investigación y Análisis, S.A. de C.V. (Mexico City).

Inventory of Agrochemicals Utilized in the Study Area

On the basis of surveys applied to agricultural laborers, we were able to compile an inventory of the agrochemicals utilized on 70 agricultural establishments devoted to cultivating chili peppers. We first obtained information from businesses on the products available in local markets for chili pepper cultivation, and then showed respondents a list of those products, asking them to identify the ones that were utilized on a daily basis.

Statistical Analysis

To evaluate whether more severe genetic damage was related to consuming well- and/or tap-water, a linear model adjusted for heterogeneity of variance was used with genetic damage (AU were obtained from the mean visual score of four slides per donor) as the response variable and frequency of consumption of well- and/or tap-water (three levels: always, sometimes, never) as the factor. This analysis was repeated taking into account age (≤40 and >40 years), occupation (conventional agriculture workers, organic agriculture workers, and workers not associated with agriculture), and consumption of alcoholic drinks and tobacco. Nonsignificant factors were removed from the model sequentially.

It is possible that the agricultural workers involved in conventional agriculture who no longer handle agricultural chemicals may have done so in earlier periods, so there could be histories of exposure that went undetected

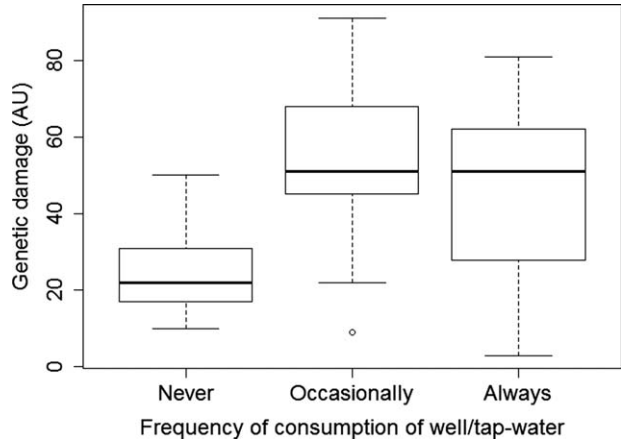

Fig. 1. Level of DNA damage (AU) observed in consumers of well- and/or tap-water in relation to frequency of consumption. Boxes show the lower (25th), median, and upper quartiles (75th). (LRT2 = 76.63, $P < 0.0001$).

in the study, but may have influenced current genetic damage. For this reason, we compared genetic damage between all conventional agricultural workers (pesticide handlers and non-pesticide handlers) and the control group. A linear model was used with the level of DNA damage as the response variable and occupation (agricultural workers vs. control group) as the factor. This analysis was repeated considering age, tobacco use and consumption of alcoholic drinks. Similarly, we evaluated whether genetic damage was more closely-associated with direct handling of agricultural chemicals. For this purpose, the control group was first compared to agricultural workers involved in conventional agriculture that handle agrochemicals, and then to those that have no direct contact with these substances. All statistical analyses were performed using R version 2.13.1 [R Development Core Team, 2008].

RESULTS AND DISCUSSION

Cellular viability, evaluated by cytotoxic analysis, was found to be high (95.59% ± 3.12; Mean ± SD). This result is important because Tice et al. [2000] recommend determining genotoxicity in organisms that manifest little or no cytotoxic damage. A significant difference in the genetic damage (AU) present in buccal cells was found to be related to the frequency with which people drank well- and/or tap-water ($LRT_2 = 76.63$, $P < 0.0001$). A post hoc Tukey test revealed differences between people who never drink well- and/or tap-water (controls) and those who do. These differences were independent of the frequency of ingestion ($P < 0.0001$) (Fig. 1). In fact, the levels of DNA damage in the consumers group were significantly higher than those of the control group (Table III).

Genetic cell damage may also be associated with such factors as age, lifestyle (e.g. smoking, alcohol consumption), and exposure to other potentially genotoxic agents (e.g. agricultural chemicals). For this reason, we repeated the analysis taking into account age, participants' occupations, and whether they were smokers or habitual consumers of alcoholic drinks. This analysis showed that upon controlling for these factors the association between genetic damage and the frequency of drinking well- and/or tap-water persisted ($LRT_2 = 16.08$, $P = 0.0003$, Table III). Therefore, the degree of genetic damage found was not related to the consumption of alcoholic drinks ($LRT_1 = 0.23$, $P = 0.63$), smoking ($LRT_1 = 2.11$, $P = 0.15$), occupation ($LRT_2 = 0.55$, $P = 0.76$), or age ($LRT_1 = 1.32$, $P = 0.25$).

TABLE III. Estimates of the Parameters and Their Associated Standard Error (SE) for Genotoxic Damage in Relation to Water Consumption

			Controlling by age, occupation and life style	
Tap water consumption	Estimate	SE	Estimate	SE
Never	24.51	1.7	28.95	8.5
Occasionally	53.82	2.9	54.50	6.3
Always	46.92	3.4	47.28	7.0

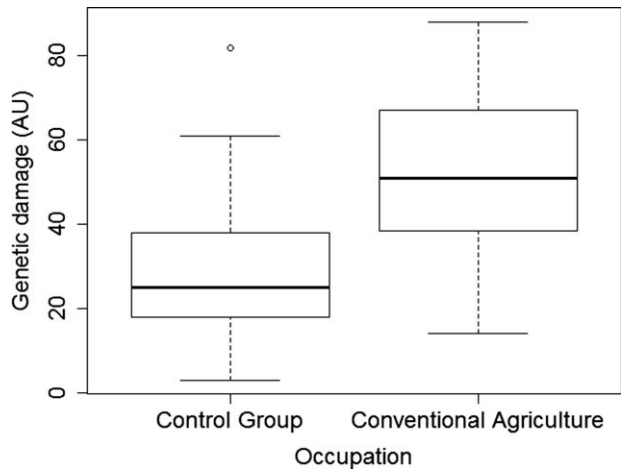

Fig. 2. Level of DNA damage observed between conventional agricultural workers and the reference group. Boxes show the lower (25th), median, and upper quartiles (75th). ($F_{(1,132)} = 51.61$, $P < 0.0001$).

A linear model was then utilized to explore differences in the level of DNA damage (AU) between the agricultural workers in conventional agriculture and the reference group (Fig. 2). Here, the agricultural workers showed greater genetic damage than controls ($F_{(1,132)} = 51.61$, $P < 0.0001$, Table IV); a difference that persisted even when the factors of age, tobacco use and consumption of alcoholic drinks ($F_{(1,129)} = 56.79$, $P < 0.0001$, Table IV) were included. The >40 group had greater genetic damage than the ≤40 group ($F_{(1,129)} = 7.51$, $P = 0.007$), but no differences related to smoking or consumption of alcoholic drinks were detected ($P > 0.5$ for both factors). This analysis was repeated to compare the reference group exclusively to the group of pesticide handlers, and then only to the group of non-pesticide handlers. The former showed greater genetic damage than controls ($F_{(1,108)} = 33.16$, $P < 0.0001$), and this difference persisted ($F_{(1,105)} = 33.83$, $P < 0.0001$) even

TABLE IV. Estimates of the Parameters and Their Associated Standard Error (SE) for Genotoxic Damage in Relation to Occupation

			Controlling for age and life style	
Occupation	Estimate	SE	Estimate	SE
Reference group	28.96	2.4	18.92	5.6
Agricultural workers	51.02	3.1	41.49	3.0

when the factor of age was considered ($F_{(1,105)} = 18.12$, $P < 0.0001$) and whether the person smoked or consumed alcoholic drinks ($P > 0.5$ for both factors). The non-pesticide handlers also showed greater genetic damage than the reference group ($F_{(1,73)} = 58.11$, $P < 0.0001$), and this difference remained when age and tobacco and alcohol consumption were accounted for ($F_{(1,70)} = 34.5$, $P < 0.0005$). These findings indicate that these factors were not associated with the level of genetic damage ($P > 0.1$ for all three factors).

The graph of the percentage frequency of the levels of genetic damage of all participants (Fig. 3) shows greater DNA alterations for the agricultural workers. Meanwhile, consumers of well- and/or tap-water with no work-related exposure to pesticides (storeowners, functionaries, etc.) presented levels of genetic damage that were similar to those of controls.

Experimental research has shown that various components of agricultural chemicals produce genotoxicity and alter genetic material [Mañas et al., 2009, Bernardi et al., 2015]. The relation between pesticides and various types of cancer (e.g., lip, stomach, brain, prostate, connective tissue, lymphatic and hematopoietic) in agricultural workers has been cited in several studies [Van Mæle-Fabry and Willems, 2004; Alavanja et al., 2005]. The concentrations of endosulfan α and β detected in the surface water were 0.30 to 13.77 mg/L and 0.55 to 9.02 mg/L, respectively (Table V). Though local residents reported that they do not drink these waters, this possibility cannot be ruled out. This situation, coupled with residents' ingestion of vegetables and meat produced in this contaminated area, may pose a risk to human health. The World Health Organization (WHO) recommended a guideline value of 20 μg/L [WHO, 2004a], a dose calculated on the basis of an Acceptable Daily Intake of 0.006 mg/kg of body weight and considering that a 60 kg adult consumes 2 L of drinking water per day. Our results exceeded the WHO recommendation.

Endosulfan has been significantly associated with such neuro-behavioral alterations as hydrocephalus, mental retardation, cognitive disorders and Parkinson's disease [Pradhan et al., 1997; Roberts et al., 2004, 2007; Wang et al., 2006; Cabaleiro et al., 2008]. It also causes adverse effects on the reproductive system [Dalsenter et al., 1999; Saiyed et al., 2003; Damgaard et al., 2006] and acts as an endocrine disruptor. Endosulfan has further been associated with precocious puberty, cervical cancer, endometriosis, recurrent abortion, and other estrogen-dependent disorders [ATSDR, 2000; Foster and Agarwal, 2002], as well as with many congenital malformations. Moreover, endosulfan shows mutagenic and genotoxic effects in human lymphocytes and liver cells [Lu et al., 2000; Bajpayee et al., 2006; Antherieu et al., 2007]. Evidence of the potential carcinogenicity of endosulfan, however, is still inconclusive due to a lack of epidemiological data. In conditions of chronic exposure, the high mortality rate in test animals caused by endosulfan's acute, elevated toxicity [ATSDR, 2015] has impeded conducting studies that obtain duly-supported results. There are reports of genotoxic damage, effects on the kinetics of the cell-cycle in mammals, promotion of exchange between sister chromatids in rat embryos, and mutagenic effects in insects; all of which strongly suggest that endosulfan is probably a mutagenic [ATSDR, 2015]. In addition, the

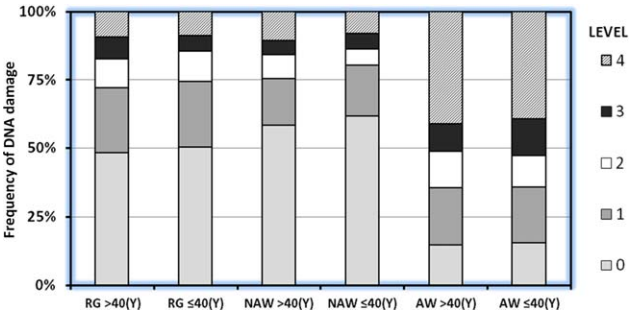

Fig. 3. Comet assay analysis in epithelial cells of consumers and non-consumers of well- and/or tap-water from Todos Santos, BCS, Mexico. The frequency of epithelial cell damage is graded in five levels of DNA breaks. RG = reference group; NAW = non-agricultural workers; AW = agricultural workers; (Y) years.

TABLE V. Organochlorine Pesticides and Heavy Metals Detected in Water From Wells, Canals, and/or Taps in Todos Santos, BCS, Mexico

Number	Place	Type	Concentration (mg/L)	Allowable level (mg/L)
4	Irrigation canals	Endosulfan α	0.30–13.77	0.20[a]
		Endosulfan β	0.55–9.02	0.20[a]
2	Agricultural wells	Arsenic	0.0061–0.0088	0.01[b]
2	Agricultural wells	Mercury	0.00030	0.001[c]
1	Well in town	Lead	0.038	0.01[d]
2	Tap in town	Lead	0.011	0.01[d]

[a]WHO [2004b].
[b]WHO [2011a].
[c]WHO [2005].
[d]WHO [2011b].

TABLE VI. Main Effects of Some Agrochemicals Used in Chili Pepper Cultivation in Todos Santos, BCS, Mexico

Commercial name	Active ingredient	Effects
CONFIDOR	Imidacloprid	Genotoxic and cytotoxic effects [Calderón-Segura et al., 2012]. Increased risk for DNA fragmentation and chromosomal aberrations [Costa et al., 2009].
PERFEXTHION	Dimethoate	Alter the genetic material particularly chromosomes in mammalian cultures [Jamil et al., 2005].
REGENT	Phenylpyrazol	Highly toxic. Induced cytotoxicity [Vidau et al., 2009].
TAMARON, AGRESOR	Methamidophos	Cytogenetic and genotoxic effects. Acetylcholinesterase inhibitor. Highly toxic to mammals [Karabay and Oguz, 2005].
THIODAN, ENDOR	Endosulfan	Endocrine-Disrupting Chemical. Estrogenic effects on Human Estrogen-Sensitive Cells [Soto et al., 1994]. Genotoxic effects [Lu et al., 2000].
RONSTAR 25	Oxadiazon	Risk of cancer with chronic exposure. Effects on liver in animals [Richert et al., 1996].
MALATION	Malathion	Potent genotoxic agent and may be regarded as a potential germ cell mutagen [Giri et al., 2002].
GRAMOXONE	Paraquat	Evidence from animal and cell models suggests that pesticides cause a neurodegenerative process leading to Parkinson's disease [Costello et al., 2009].
CABRIO C	Nicotinamide	Potent Inducer of Endocrine Differentiation in Cultured Human Fetal Pancreatic Cells [Otonkoski et al., 1993]
FAENA, CENTELLA	Glyphosate	Endocrine disruptors in human cell lines [Gasnier et al., 2009]. Induce Apoptosis and Necrosis in Human Umbilical, Embryonic, and Placental Cells [Benachour and Séralini, 2009]

Stockholm Convention includes it on the United Nations' list of Persistent Organic Contaminants (POC) that should be banned worldwide [UNEP, 2011].

The surveys conducted among agricultural producers and laborers on the utilization of agrochemicals allowed us to obtain detailed information on the active substances used in the study area, and so identify those that are considered carcinogenic, mutagenic, and responsible for alterations in fetal development and fertility, among others (Table VI). All these substances, except endosulfan, are not organochlorine pesticides and therefore have not been detected by the present study.

We found arsenic (0.0061–0.0088 mg/L) and mercury residues (0.00030 mg/L) in three wells, but in concentrations below the limits allowed by Mexican norms (Table V). The affected wells are located on the banks of a stream, which originates in an ancient mining zone (Valle Perdido, El Triunfo). Water from these wells is not used for human consumption, but the possibility that agricultural laborers drink it cannot be discarded. Also, these wells are used to irrigate crops, and so could constitute a risk for human health through ingestion of arsenic-contaminated foods. Lead was also found (0.011–0.038 mg/L) and its origin is probably from old plumbing pipes or other unknown origin.

In our work, neither consumption of alcoholic drinks nor smoking was associated with greater genetic damage. Some studies have proven that consuming tobacco and alcohol causes genotoxicity [Riedel et al., 2003; DeMarini, 2004], and that both chronic consumption and the amount consumed affect the degree of such damage. In the case of our participants, it was not possible to obtain clear, precise information on the amount and/or frequency of consumption of these substances, so the categorization employed (smoker/drinker vs. non-smoker/non-drinker) is not necessarily representative of chronic exposure to these agents. It is possible, as well, that other factors—such as age or occupation (i.e., exposure and pesticide-handling)—confounded the effect of consuming tobacco or alcoholic drinks on genetic damage.

DNA damage is commonly considered a marker of the risk of developing cancer (due to the evident implication of DNA lesions in mutagenesis). However, in contrast to chromosome aberrations and micronuclei, which have been shown to have predictive value for cancer, there is no evidence that a high level of DNA damage in epithelial or blood cells indicates a risk of developing cancer. Because these kinds of DNA damage are repaired quickly and the lesions involved are only transitory, they should be taken more as markers of exposure than markers of effects. The repairing of the DNA damage eliminates the potential for mutagenesis caused by the changes in the DNA, and is a crucial element in protecting against cancer. Individual differences in the ability to repair such damage will affect people's susceptibility to cancer. It is probable that genetics, the environment, lifestyle, and individual nutritional influences are factors that affect different people's capacity to repair DNA [Collins and Dusinska, 2009].

CONCLUSION

A significant difference was found in genetic damage through an evaluation using the comet assay that compared consumers and non-consumers of well- and/or tap-water. The increased genetic damage observed in consumers of well- and/or tap-water is related to the work activities (agricultural laborers) of the subjects in the group that consumed those waters. However, we cannot rule out the possible presence of other metabolites in the water that went undetected by the chromatographic techniques utilized, especially in light of the wide variety of harmful and toxic products whose use in agricultural activities in

the study area was confirmed during this work. The results obtained do, however, demonstrate a clear relation between genetic damage and exposure to pesticides. The use of the comet assay as a biomarker of pesticide exposure confirms, once again, its usefulness. In addition, during this study we were able to confirm the utilization of products that are considered carcinogenic or/and mutagenic that have a significant impact on human health and so demonstrate, once more, the work-related risks for human health caused by pesticide exposure. We recommend expanding the analytical capacities in the detection of chemicals released to the environment by the different anthropogenic activities.

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AUTHOR CONTRIBUTIONS

C.V.B. designed and coordinated the study after its approval by the Board of Ethics at the Research Institute for Biomedical Research, National Autonomous University of Mexico (UNAM). C.V.B., L.M., and paramedics of the Fire Department of Todos Santos sampled buccal cells and conducted surveys to collect information on lifestyle habits and agrochemical exposure of the selected participants. C.V.B., M.R.O., V.S.P., G.A., L.C., and D.D. standardized the comet assay protocol and measured DNA damage in buccal cells. L.L.-C. performed the statistical analysis. C.V.B., L.L.-C., and L.M. analyzed the data. The manuscript was written by C.V.B. with important contributions by L.L.-C. and L.M. All authors approved the final version of the manuscript.

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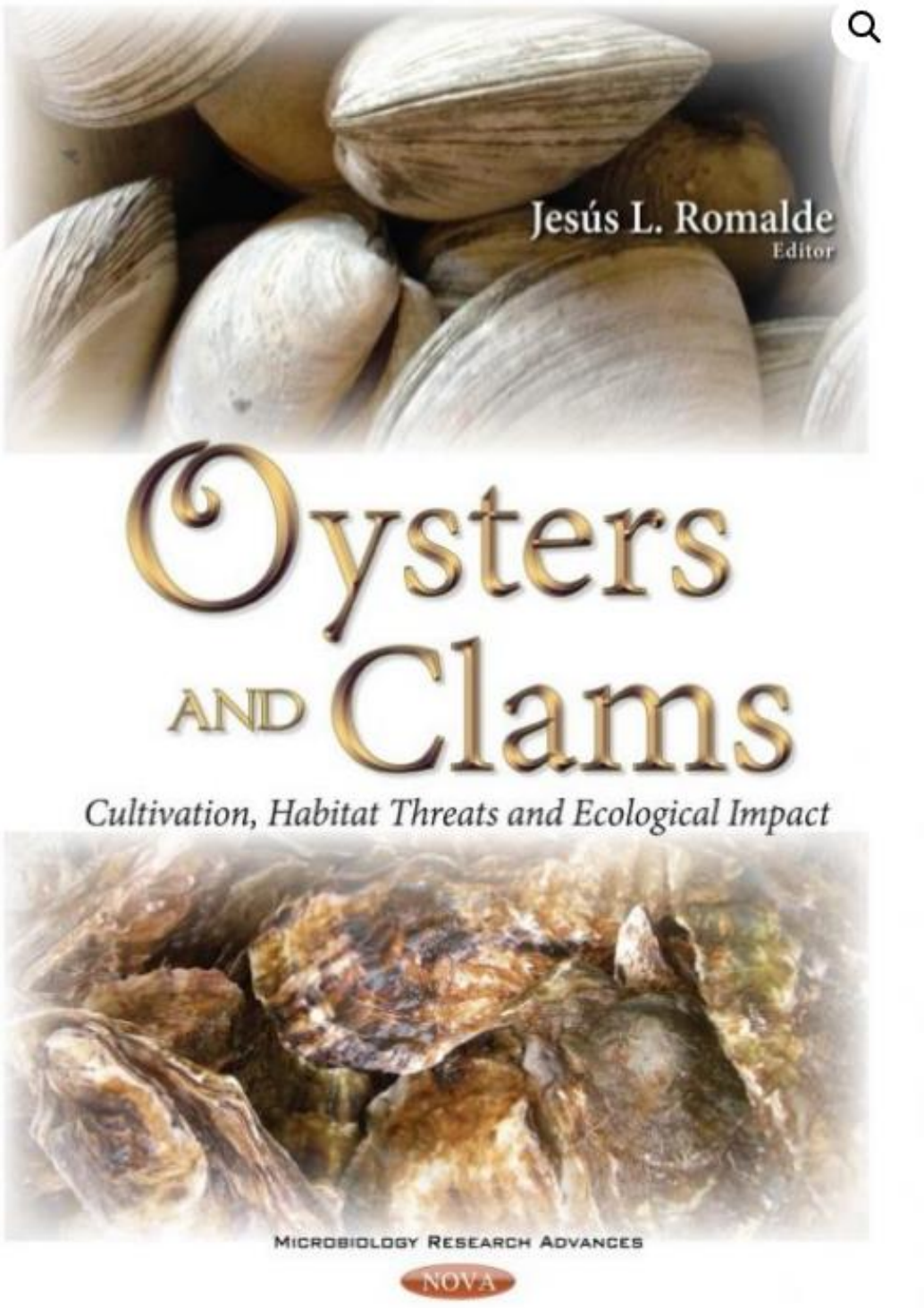

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