
# UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

DOCTORADO EN CIENCIAS DE LA PRODUCCIÓN Y DE LA SALUD ANIMAL

## Mecanismo de acción y toxicidad de nuevos carbamatos con efecto sobre garrapatas *Rhipicephalus microplus* (*Boophilus microplus*)

**T E S I S**

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*Dedico esta tesis con todo mi amor a*

*mis padres: Gilberto Prado e Isabel Ochoa,*

*a mi amor: Francisco Pérez Gallegos,*

*y*

*a mis hijos: Francisco, Eduardo y Ana Paula*

*Gracias por todo su apoyo, comprensión y amor.*

*Lupita*

"Cuando no puedas correr trota, cuando no puedas trotar camina, cuando no puedas caminar usa el bastón, pero nunca te detengas".

*Madre Teresa de Calcuta*

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# Abreviaturas

| | |
|---|---|
| **A** | Albúmina |
| **AChE** | Acetilcolinesterasa |
| **AIT** | Prueba de inmersión de adultas |
| **ALT** | Alanina aminotransferasa |
| **AST** | Aspartato aminotransferasa |
| **c** | Corion |
| **CHE** | Colinesterasa |
| **$CIE_{99}$** | Concentración de inhibición de eclosión 99% |
| **Cl** | Cloro |
| **dgv** | Vesícula germinal degenerada |
| **dL** | Decilitros |
| **$DL_{50}$** | Dosis letal 50% |
| **DMSO** | Dimetilsulfóxido |
| **dn** | Nucleolo degenerado |
| **FES** | Facultad de Estudios Superiores |
| **G** | Globulina |
| **g** | Gramos |
| **GABA** | Acido gamma amino butírico |
| **GGT** | Gammaglutamil transferasa |
| **GHS** | Globally Harmonized System |
| **gv** | Vesícula germinal |
| **h** | Horas |
| **ic** | Corion incompletamente depositado |
| **IMI** | Inhibición del índice mitótico |
| **io** | Ovocitos inmaduros |
| **$k_2$** | Constante de carbamilación |
| **$k_d$** | Constante de disociación |
| ***Kdr*** | Knock down resistance |
| **kg** | kilogramos |
| **$k_i$** | Constante de reacción bimolecular |

| | |
|---|---|
| **$K_m$** | Constante de Michaelis-Menten |
| **L** | Litros |
| **$LC_{99}$** | Concentración letal 99% |
| **$LD_{50}$** | Dósis letal 50% |
| **LDH** | Lactato deshidrogenasa |
| **LPK** | Cinética de proliferación de linfocitos |
| **LPT** | Prueba de paquete de larvas |
| **M** | Molar |
| **M1** | Primera división mitótica |
| **M2** | Segunda división mitótica |
| **M3** | Tercera división mitótica |
| **mg** | Miligramos |
| **MI** | Índice mitótico |
| **Mictr** | Índice mitótico del cultivo control-DMSO |
| **MiIob** | Índice mitotic del cultivo tratado |
| **mL** | Mililitros |
| **mM** | Milimolar |
| **MN** | Micronúcleo |
| **MN-NCE** | Eritrocitos normocromáticos micronucleados |
| **MN-PCE** | Eritrocitos policromáticos micronucleados |
| **mo** | Ovocito maduro |
| **n** | Nucleolo |
| **Na** | Sodio |
| **nm** | Nanómetros |
| **nmol** | Nanomoles |
| **NOAEL** | Nivel sin efectos adversos observados |
| **NOM** | Norma Oficial Mexicana |
| **oe** | Epitelio ovárico |
| **OECD** | Organización para la Cooperación y el Desarrollo Económico de los Estados |
| **ov** | Oviducto |
| **p.t.** | Pos-tratamiento |
| **PCE** | Eritrocitos policromáticos |

| | |
|---|---|
| **PHA** | Fitohemaglutinina |
| **pn** | Núcleo picnótico |
| ***R. microplus*** | *Rhipicephalus microplus* |
| **RI** | Índice de replicación |
| **s** | grupo satélite |
| **SAGARPA** | Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación |
| **SEM** | Microscopía electrónica de barrido |
| **TBARS** | Sustancias reactivas al ácido tiobarbitúrico |
| **UNAM** | Universidad Nacional Autónoma de México |
| **v** | Vacuola |
| $V_{max}$ | Velocidad máxima |
| **w/v** | Peso/volumen |
| **µg** | Microgramos |
| **µL** | Microlitros |

# Índice

# Resumen

En este trabajo se evaluó el efecto de nuevos metil o etil carbamatos diseñados y sintetizados en FES Cuautitlán sobre *Rhipicephalus microplus,* se estudió el mecanismo de acción de los carbamatos con efecto sobre *R. microplus* y su toxicidad en mamíferos. De los resultados obtenidos en este trabajo se escribieron cinco artículos de investigación que se encuentran en diferentes etapas de su publicación en revistas científicas internacionales y se registró una patente del uso de los compuestos como antiparasitarios.

En el primer artículo se evaluó el efecto de 17 nuevos carbamatos sobre adultas y larvas de *R. microplus* por medio de las pruebas de inmersión de hembras adultas y de paquete de larvas. En este estudio se mostró en general que seis carbamatos inhibieron la oviposición hasta en un 65.4% e inhibieron la eclosión hasta en un 100% ($p<0.05$). Los huevos ovipositados por las garrapatas tratadas se observaron disgregados, oscuros, secos y no fueron viables.

En el segundo artículo se evaluó el efecto del etil-4-bromofenil-carbamato (LQM 919) y etil-4-clorofenil-carbamato (LQM 996) sobre la actividad de acetilcolinesterasa (AChE), estructura de los huevos y órganos reproductores de dos cepas de *R. microplus.* Los resultados de este estudio mostraron que los efectos de estos carbamatos sobre *R. microplus* son independientes a la inhibición de la AChE como ocurre con otros ixodicidas y mostraron que las alteraciones morfológicas observadas en los órganos reproductores de la garrapata fueron debidas a la acción de estos carbamatos sobre la vitelogénesis y viabilidad de las células del ovario.

En el tercer artículo se determinó la toxicidad oral aguda y dérmica aguda de ambos carbamatos en ratas. La $DL_{50}$ oral fue de 300-2000 mg/kg y la dérmica de >5000mg/kg para ambos carbamatos, se presentaron algunos signos de toxicidad en la exposición oral, en la exposición dérmica no se observaron signos de toxicidad en ninguna de las dosis empleadas. Se demostró que los carbamatos evaluados son de baja toxicidad oral y dérmica.

En el cuarto artículo se evaluó la toxicidad subcrónica (90 días) en ratas de los carbamatos LQM 919 y LQM 996. En este estudio se observaron alteraciones en algunos de los parámetros evaluados como el hematocrito, porcentaje de reticulocitos, algunas enzimas hepáticas y creatinina en las ratas expuestas a los carbamatos y se demostró la reversibilidad de estas alteraciones al suspender la

exposición a los carbamatos. Se determinó como dosis sin efectos adversos observables (NOAEL) la dosis de 12.5 mg/kg/día.

Finalmente, en el quinto artículo se evaluó el daño genético *in vivo* producido por los carbamatos evaluados por medio de la prueba de micronúcleos en sangre periférica de rata, además se evaluó el efecto de los carbamatos *in vitro* sobre la cinética de proliferación celular en cultivos de linfocitos humanos. Los resultados de este estudio mostraron el potencial genotóxico de los carbamatos estudiados en algunas dosis empleadas, así como su efecto sobre el ciclo celular.

Con los resultados de esta tesis se demostraron la eficacia de los nuevos carbamatos sobre *R. microplus*, el mecanismo de acción diferente a los ixodicidas comerciales y su baja toxicidad en mamíferos. Lo anterior hace factible el desarrollo de nuevas formulaciones farmacéuticas con estas nuevas moléculas para el control de garrapatas. Sin embargo, de acuerdo a los efectos tóxicos observados, se sugiere precaución y continuar con el estudio de los riesgos a la salud.

# Abstract

This study evaluated the effect of new methyl or ethyl-carbamates designed and synthesised in FES Cuautitlán UNAM on *Rhipicephalus microplus* ticks. We studied the mechanism of action of the carbamates with effect on *R. microplus* and its toxicity in mammals. With the results obtained in this work were written five research papers that are in various stages of their publication in international scientific journals and was registered a patent of the use of these carbamates as antiparasitic compounds.

In the first research paper, the effects of 17 new carbamates on adults and larvae of *R. microplus* were evaluated by adult immersion test and larval packet test. This study showed that six of the new carbamates inhibit the oviposition until in a 65.4% and inhibit until 100% of larvae hatching ($p < 0.05$). The eggs oviposited by treated ticks were dark, dry and they were not viable.

In the second research paper, the effects of ethyl-4-bromophenyl-carbamate (LQM 919) and ethyl-4-chlorophenyl-carbamate (LQM 996) on the activity of acetylcholinesterase (AChE), structure of eggs and reproductive organs of two strains of *R. microplus* were evaluated. The results of this study showed that the effects of these carbamates on *R. microplus* are independent to the inhibition of AChE and showed that the morphological alterations observed in the reproductive organs of the treated ticks were due to the action of these carbamates on the vitellogenesis and viability of ovary cells.

In the third research paper, the acute oral and dermal toxicity of both carbamates in rats were evaluated. The oral $LD_{50}$ was found between 300 to 2000 mg/kg and the dermal $LD_{50}$ was found > 5000 mg/kg for both carbamates. There were some signs of toxicity in rats treated by the oral pathway; in the dermal exposure were no signs of toxicity at any of the doses employed. This study show that these carbamates are of low oral and dermal toxicity.

In the fourth research paper, the subchronic toxicity (90 days) in rats of the carbamates LQM 919 and LQM 996 was assessed. Alterations in some of the evaluated parameters as haematocrit, reticulocyte percentage, some liver enzymes and plasma creatinine in rats exposed to carbamates were observed in this study and showed the reversibility of these changes when the exposure to carbamates was suspended.

Finally, in the fifth research paper, the *in vivo* genetic damage produced by the carbamates evaluated through testing of micronuclei in peripheral blood of rat was assessed; also we evaluated the *in vitro* effect of carbamates on cell proliferation kinetics in human lymphocyte cultures.The results of this study showed the genotoxic potential of the studied carbamates at some of the doses employed, as well as its effect on the cell cycle.

The results of this thesis demonstrated the efficacy of the new carbamates on *R. microplus*, its mechanism of action wich is different from commercial ixodicides and its low toxicity in mammals.This makes possible the development of new pharmaceutical formulations with these new molecules for the tick control. However, according to the toxic effects that were observed, we suggest caution and to continue with the study of the health risks.

# Capítulo 1

## INTRODUCCIÓN

Las garrapatas son los ectoparásitos hematófagos más importantes del ganado en las áreas tropicales y subtropicales del mundo (Baxter y Barker, 1998). *Rhipicephalus microplus* es la especie de garrapata más importante en estas áreas ya que produce grandes pérdidas económicas para la ganadería. Los efectos por la infestación de garrapatas incluyen: anemia, reducción en crecimiento, reducción en parámetros reproductivos, reducción de la producción de carne y leche, disminución de la calidad de las pieles, parálisis y transmisión de enfermedades como babesiosis y anaplasmosis (Oliveira et al., 2005; Rodriguez-Vivas et al., 2007a; Solorio-Rivera et al., 1999). La estrategia más utilizada para el control de garrapatas ha sido el uso de ixodicidas químicos, sin embargo, el uso intensivo de estos ha ocasionado el desarrollo de poblaciones de garrapatas resistentes (George et al., 2004; Perez-Cogollo et al., 2010; Rodriguez-Vivas et al., 2007b), La resistencia de *R. microplus* a los ixodicidas es un problema para la ganadería mundial. En México, tiene gran importancia porque su distribución coincide con las áreas tropicales, que son las zonas más importantes de la industria ganadera nacional (Soberanes-Céspedes et al., 2002). El desarrollo de nuevos ixodicidas seguramente contribuirá a reducir el problema de resistencia y reducirá las enormes pérdidas económicas producidas por la infestación de garrapatas.

## 1. GENERALIDADES DE *Rhipicephalus microplus*

*R. microplus* es originaria del sureste de Asia, se ha distribuido a través de los trópicos incluyendo Australia, Este y Sur de África, y Centro América. Fue introducida a México junto con *R. annulatus,* por el sur de Estados Unidos de Norte América, antes de ser exitosamente erradicada de dicho país (Jongejan y Uilenberg, 2004).

*R. microplus* (antes llamada *Boophilus microplus*) actualmente está reclasificada por su relación filogenética. Pertenece al *phylum*: *artropoda*, clase*: arachnida*, orden*: acarina,* suborden*: metastigmata,* familia*: ixodidae* (Barker y Murrell, 2004).

Su cuerpo o idiosoma es de forma ovalada, presenta un escudo en forma de lengüeta color café, el cual es corto en hembras y largo en machos, el gnatosoma se sitúa en la región anterior del cuerpo (figura 1). El gnatosoma comprende las partes bucales de la garrapata. Está formado por un par de palpos con cuatro segmentos, un par de quelíceros esclerotizados y bisegmentados, y el hipostoma en la parte media del gnatososma. La base del gnatosoma es de forma hexagonal (Soulsby, 1987).

Los estadios de desarrollo de esta garrapata, se caracterizan porque las larvas poseen tres pares de patas (hexápodas), carecen de placas estigmales y orificio genital. Las ninfas y adultos poseen cuatro pares de patas, sin embargo, las ninfas carecen de abertura genital y áreas porosas, a diferencia de los adultos (Soulsby, 1987).

Figura 1. *Rhipicephalus microplus*

*R. microplus* presenta un ciclo de vida monoxeno, que se caracteriza por la utilización de un solo hospedador para completar su desarrollo, que comprende un periodo de entre 4 y 10 meses, siendo el hospedador generalmente un bovino. El ciclo biológico de *R. microplus* se compone de tres fases: La fase no parásita o de vida libre, la fase de encuentro y la fase parásita (figura 2). La fase no parásita comprende desde que la hembra alimentada se desprende del hospedador, cae a la vegetación y por fototaxia negativa busca lugares protegidos donde se lleva a cabo la oviposición, cada hembra puede ovipositar entre 3500 y 4400 huevos, el período de preoviposición es de 2 a 39 días y el de oviposición de 4 a 44 días, dependiendo de las condiciones medioambientales. Después de la oviposición sigue el período de incubación de los huevos el cual es de 14 a 146 días, seguido de la eclosión de las larvas (Soulsby, 1988). Las larvas se alimentan de la sustancia vitelina del huevo (Estrela et al., 2007) y suben a la punta de los pastos para encontrar a su hospedero en la fase de encuentro. La fase parásita incluye diferentes eventos: adherencia a la

piel o pelaje del hospedador, búsqueda en la piel de un sitio adecuado, inserción de las partes bucales en la epidermis y dermis del hospedador, fijación de la larva y alimentación. Durante la alimentación se lleva a cabo la muda de larva a ninfa, de ninfa a adultos y la fecundación. Esta fase tiene una duración de 21 días en promedio, algunos investigadores mencionan que se pueden encontrar ninfas entre el día 5° al 14° y adultos entre el día 13° y 25° a partir de la fijación de las larvas. Finalmente las hembras repletas o ingurgitadas se desprenden y caen del hospedador (Anderson y Magnarelli, 2008).

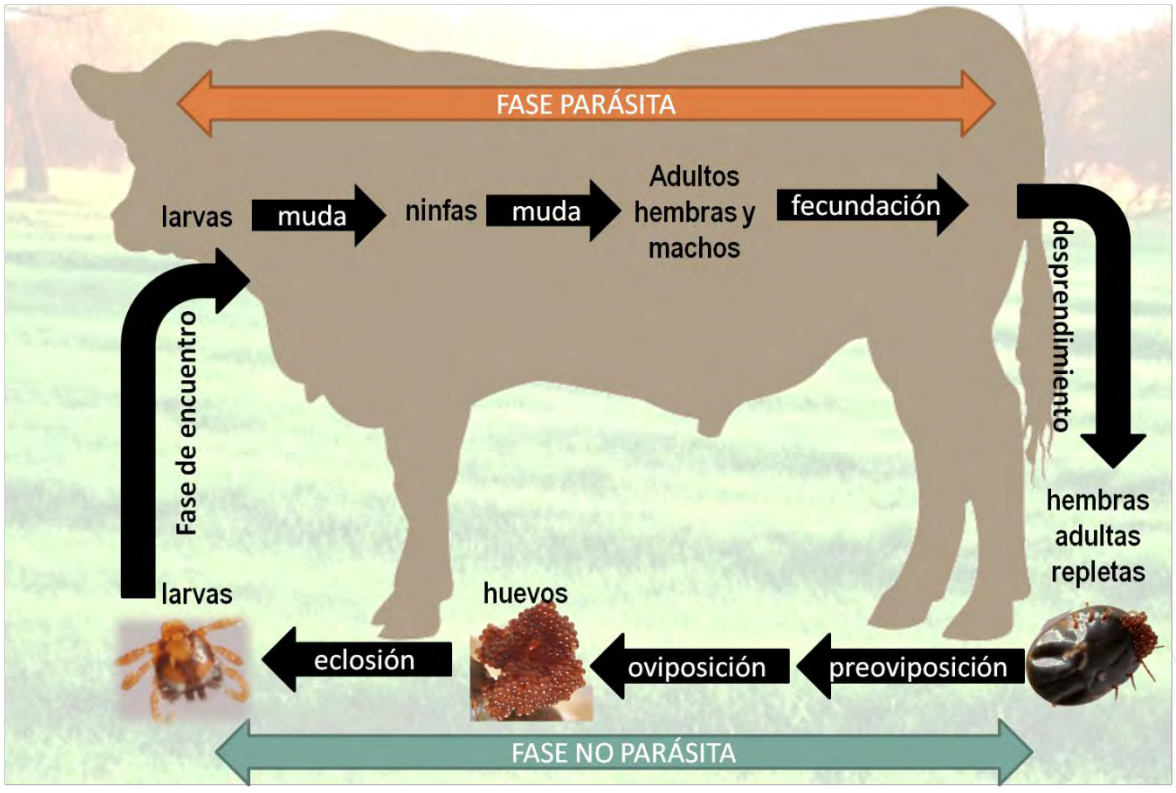


Figura 2. Ciclo biológico de *Rhipicephalus microplus*

## 1.1 Control de garrapatas

Los métodos actuales para el control de la garrapata implican el uso de métodos químicos y no químicos, y la aplicación sistemática de dos o más métodos (manejo

integrado de plagas). Aunque el control de las garrapatas en bovinos se basa principalmente en el uso de productos químicos sobre el cuerpo de éstos a intervalos específicos (Ortiz, 1999). El historial del uso de ixodicidas en el mundo incluye diferentes productos como los arsenicales, organoclorados, organofosforados, carbamatos, amidinas cíclicas, fenilpirazolonas, piretroides e ivermectinas. Existen métodos alternativos como el inmunológico, por medio de la utilización de una vacuna elaborada con metodología de biología molecular de una proteína de los intestinos de las garrapatas (antígeno Bm86), sin embargo, esta vacuna ha mostrado una efectividad menor que los ixodicidas. También se han utilizado algunas leguminosas y pastos tropicales que tienen un efecto anti-garrapata, y la utilización de hongos entomopatógenos de garrapatas (Fernández Ruvalcaba et al., 2012), pero su aplicabilidad para el control de garrapatas a nivel de campo no ha sido determinada, por lo que el uso de ixodicidas químicos sigue siendo la estrategia de elección.

### 1.1.1. Control Químico

En la actualidad se emplean diversas familias de ixodicidas dentro de las cuales se encuentran:

*Organofosforados***:** Los organofosforados son esteres orgánicos del acido fosfórico y sus derivados. Estos son clasificados como los pesticidas más tóxicos para los vertebrados y están relacionados con gases neurotóxicos. etión, chlorpyriphos, chlorfenvinphos y coumaphos son cuatro de los organofosforados más usados mundialmente para el tratamiento de ganado infestado con garrapatas (George et al., 2004; Rajput et al., 2006). Los organofosforados son inhibidores irreversibles de la acetilcolinesterasa (AChE), provocan alteraciones en distintos eventos del sistema nervioso central y la muerte del artrópodo (Fournier y Mutero, 1994).

*Formamidinas:* Estos fármacos fueron desarrollados en el año de 1970. Las amidinas son antagonistas de los receptores de la octopamina en el sistema nervioso

de los parásitos: provocan hiper-excitabilidad y seguidamente parálisis y muerte. La excitación provoca además que las garrapatas no logren fijarse al hospedador. También se ha atribuido su efecto a la inhibición de monoamino oxidasas (Atkinson et al.1974, Schuntner y Thompson 1976). En esta familia se encuentra el amitraz, clordimeform, clorometiurom y clenpirina (Botana López et al., 2002; George et al., 2004).

*Piretroides***:** Los piretroides naturales, que son muy costosos e inestables a la luz solar, son los predecesores de una serie de productos sintéticos, los piretroides sintéticos. Los piretroides tienen una historia de evolución que comenzó en 1949, pero la tercera generación de estos químicos, permetrina y fenvalerato, fueron los primeros disponibles para el control de garrapatas en el ganado. Cypermethrina, deltamethrina y cyhalothrina son ejemplos de la cuarta generación de piretroides ciano-sustituidos o piretroides tipo II que son ixodicidas efectivos (George et al., 2004). Estos actúan a nivel nervioso central uniéndose a los canales de sodio y retardando su cierre, lo que ocasiona la entrada prolongada de sodio provocando descargas repetitivas en los nervios e hiperexitabilidad causando parálisis y muerte en los artrópodos (Tan et al., 2005; Shafer et al., 2005).

*Lactonas macrocíclicas*: Las lactonas macrocíclicas son endectocidas eficientes en el control de endo y ectoparásitos. Existen dos clases de lactonas macrocíclicas con actividad ixodicida, las avermectinas que son derivadas del actinomiceto *Streptomyces avermitilis* y las milbemycinas que son derivadas de productos de fermentación de *S. higroscopicus aureolacrimosus*. Ivermectina, doramectina y eprinomectina son derivadas de las avermectinas, mientras que la moxidectina es la única lactona macrocíclica derivada de la milbemycina utilizada como ixodicida (George et al., 2004). Las lactonas macrocíclicas producen su efecto antiparasitario al incrementar la permeabilidad de la membrana celular por los iones de cloro ($Cl^-$), con la consecuente hiperpolarización y parálisis de la musculatura faríngea y somática de los parásitos (Rodríguez-Vivas et al., 2010).

*Fenilpirazoles:* Son una clase química de insecticidas y acaricidas cuyo representante principal es el fipronil, introducido como ectoparasiticida en 1993, primero como pulguicida y garrapaticida para las mascotas y después en el mercado ganadero como garrapaticida y mosquicida. Este fármaco actúa como antagonista del GABA fijándose al receptor del canal interno del cloro, induciendo hiperexitabilidad del parásito. (Botana López et al., 2002; George et al., 2004).

*Inhibidores de quitina:* Estos productos interfieren en la síntesis y depósito de la quitina, inhibiendo la ecdisis o muda del parásito, generándole la muerte por deshidratación; en este grupo se puede encontrar al fenoxicarb y fluazurón (Botana López et al., 2002).

### 1.1.2 Métodos de aplicación de ixodicidas

Se emplean diferentes métodos para la aplicación de ixodicidas en el ganado bovino, estos métodos incluyen el baño de inmersión, de aspersión, la aplicación epicutánea y la aplicación parenteral.

En el baño de inmersión, se sumerge al ganado en la solución ixodicida, en general es un método altamente efectivo para el tratamiento de animales con ixodicidas, sin embargo, su inmovilidad, alto costo inicial de construcción y el costo de los productos, puede hacerlos poco prácticos para las pequeñas ganaderías (George et al., 2004).

El baño de aspersión consiste en la aplicación del ixodicida mediante el uso de una bomba de rociado, asegurándose que el ixodicida se disperse por toda la superficie corporal del animal (NOM-019-ZOO-1994).

La aplicación epicutánea, también llamada “pour-on”, consiste en la aplicación del ixodicida directamente sobre la piel del animal, por la línea media dorsal, desde la cruz hasta la región coccígea. El ixodicida actúa después de

dispersarse sobre la superficie del animal o después de absorberse a través de la piel y ser ingerido por el artrópodo (NOM-019-ZOO-1994).

La aplicación parenteral consiste en la inyección parenteral del fármaco, que al absorberse y alcanzar niveles adecuados, tiene efecto sobre las garrapatas que se encuentran alimentándose (NOM-019-ZOO-1994).

### 1.2. Resistencia a los ixodicidas

El control de garrapatas en México como en otras partes del mundo, se ha basado históricamente en el uso de diversas familias químicas de ixodicidas tales como arsenicales, organoclorados, organofosforados, carbamatos, piretroides sintéticos, amidinas e ivermectinas (George et al., 2004), el uso indiscriminado de estos productos químicos ha ocasionado la aparición de poblaciones de garrapatas resistentes y la ineficiencia sistemática de los ixodicidas (Rosado-Aguilar et al., 2008).

La resistencia a los ixodicidas se define como la habilidad de una población de parásitos, para tolerar dosis de tóxicos que resultarían letales para la mayoría de los individuos en una población normal (susceptible) de la misma especie (WHO, 1957).

Se ha reportado el fenómeno de resistencia desde los años 40’s y 50’s en Australia, Sudamérica y África, en que aparecieron poblaciones de garrapata resistentes a diversos compuestos organoclorados, a finales de los 50’s y principios de los 60’s se presentó en Australia y Sudáfrica resistencia a compuestos inhibidores de las colinesterasas como carbamatos y organofosforados. Para finales de los años 70’s ya se habían reportado en Australia cepas de garrapatas resistentes a amidinas y para inicios de los 80’s se tenía conocimiento de la resistencia a los piretroides en Australia (Soberanes-Céspedes et al., 2002).

En México se ha documentado la resistencia de *R. microplus* hacia ixodicidas organoclorados y organofosforados desde 1981, en que se caracterizó la cepa "Tuxpan" colectada en la zona del Golfo de México en la región de Tuxpan, Veracruz (Aguirre y Santamaría, 1986). Posteriormente en 1984 se identificó otra cepa denominada "Tempoal" resistente a organofosforados y clorados. En 1993, ocho años después de autorizado el uso de piretroides en México, fueron detectados los primeros casos de resistencia hacia estos ixodicidas, en el este de la República, en la región del Golfo de México donde se caracterizaron las cepas "San Jorge" y "La Mora" con resistencia a los piretroides (flumetrina, deltametrina y cipermetrina) y con mediana resistencia a organofosforados (Ortiz et al., 1995). Posterior al establecimiento de resistencia a piretroides, se incrementó considerablemente el uso del amitraz ya que este ixodicida mostró ser efectivo para el control de cepas resistentes a piretroides. A principios del año 2001 se detectó una cepa de garrapatas denominada "San Alfonso" que presentó características de resistencia a amidinas, organofosforados y piretroides sintéticos. Recientemente, Pérez-Cogollo et al. (2010) informaron por primera vez la resistencia a la Ivermectina en poblaciones de *R. microplus* en México y Miller et al. reportaron resistencia al fipronil en cinco cepas de Tamaulipas, México (2013).

Según Fragoso y Soberanes (2001), el establecimiento de la resistencia en alguna región pasa por tres fases:

1. *Fase de establecimiento*: en esta fase se selecciona el alelo resistente en una población, habitualmente este proceso se efectúa por mutaciones naturales y en forma independiente a la presión de selección.
2. *Fase de desarrollo*: en esta fase se presenta el aumento en el número de individuos resistentes de cómo resultado la sobrevivencia preferencial de los primeros sobre los individuos susceptibles después del uso de productos químicos. En este proceso pueden seguirse dos modos de selección: rápida, que ocurre cuando el gen que confiere resistencia es dominante o

parcialmente dominante y permite la selección de heterocigotos y lenta, cuando los alelos son recesivos.

3. *Fase de emergencia*: ocurre por una elevada presión de selección, es una fase corta y el alelo resistente es lo suficientemente común en la población para manifestar una reducción de la eficacia del ixodicida.

Se han reportados dos mecanismos de resistencia a los ixodicidas, estos son: detoxificación metabólica y/o modificación del sitio blanco (Rosario-Cruz et al., 2009). La detoxificación metabólica es predominantemente causada por la actividad elevada de enzimas agrupadas en familias multigénicas, tales como: citocromo P450, glutatión-s-transferasas y carboxilesterasas, las cuales degradan y/ó secuestran las moléculas de pesticidas, dependiendo de cual sistema enzimático ó familia de pesticidas esté involucrada (Dominguez-García et al., 2009). La insensibilidad en el sitio blanco se puede producir por mutaciones en: el canal de sodio, la AChE, el receptor GABA o los genes en los que están codificados los receptores de octopamina. Estos mecanismos, solos o en combinación, confieren la resistencia a todas las clases conocidas de ixodicidas disponibles (Alonso-Díaz et al., 2006).

*Resistencia a organofosforados:* Se ha reportado que un importante mecanismo de resistencia a organofosforados en insectos, son las mutaciones puntuales en el gen estructural en el que se codifica la AChE que dan por resultado la producción de una enzima cuya función está modificada como resultado de una, sustitución de una glutamina por una arginina en el gen de la AChE 3 (Temeyer et al., 2006). Un segundo mecanismo de la resistencia a los organofosforados está ligado a un aumento en la actividad de la citocromo monoxigenasa P450 (Baffi et al., 2007).

*Resistencia a piretroides*: La mutación en el gen del canal de sodio, le confiere al canal la incapacidad de unión con el pesticida, inhibiendo de esta manera su efecto tóxico, debido a la modificación estereoquímica de la estructura del canal

de sodio (Jamroz et al., 2000). A este mecanismo se le conoce como resistencia tipo *Kdr* por su denominación en inglés (knock down resistance). En *R. microplus* se ha documentado la substitución de una fenilalanina por una isoleucina en el gen del canal de sodio de cepas mexicanas, localizada en el dominio III del segmento 6 (III-S6) , la cual confiere resistencia a la familia de los piretroides sintéticos (He et al., 1999). Adicionalmente una esterasa metabólica específica con actividad de hidrolizar la permetrina, la CzEst9, se ha asociado a una alta resistencia a la permetrina (Jamroz et al., 2000).

*Resistencia a fenilpirazolonas*: Se ha reportado la presencia de mutaciones del gen GABA en *Drosophila melanogaster* (Hemingway et al., 2004), sin embargo, al presente, no se ha aislado ningún gen GABA de *R. microplus*. Por otro lado, cepas de *R. microplus* recolectadas al norte de México resultaron ser resistentes a fipronil. Los autores concluyeron que la resistencia a fipronil parece ser debido en parte a la actividad de una esterasa elevada (CzEst9), que fue preseleccionada por el uso generalizado de permetrina en la década de 1980 en México (Miller et al., 2013). Sin embargo, se deben realizar más trabajos para conocer los verdaderos mecanismos de resistencia del fipronil (Rodríguez Vivas et al., 2012).

*Resistencia al amitraz*. Existe evidencia de que el sitio de acción de las formamidinas (amitraz) es el receptor octopamina. Chen et al. (2007) fueron los primeros en describir las mutaciones de un gen putativo del receptor octopamina en *R. microplus* resistente al amitraz. El descubrimiento de estas mutaciones sólo en garrapatas resistentes al amitraz, proporciona la primera evidencia de la posibilidad de que el sitio de acción del ixodicida este modificado, como un mecanismo de resistencia al amitraz en *R. microplus* (Rodríguez Vivas et al., 2012).

## 2. CARBAMATOS

Los carbamatos son compuestos moleculares sencillos derivados inicialmente de la fisostigmina (*Physostigma venenosum*), en la actualidad estos compuestos son de origen sintético (Gupta, 2006). Formalmente los carbamatos son el éster de un alcohol (ROH) y un ácido carbámico (monoamina del acido carbónico). El ácido carbónico como tal, no existe, porque se descarboxila espontáneamente dando $CO_2$ y $NH_3$, en cambio si existen sus sales, los carbamatos, que son cristalinas y estables a temperatura ambiente (Odilón, 1993).

La historia de los carbamatos se remonta al año de 1840 cuando fue llevada a Inglaterra la planta *Physostigma venenosum* proveniente del trópico Africano, la cual era utilizada para la cacería de brujas (Gupta, 2006). Veinticuatro años después (1864), se descubre que los efectos tóxicos provocados por el frijol de Calabar son reversibles al tratar a niños intoxicados con esta semilla utilizando como antídoto atropina, eméticos y agua caliente. En ese mismo año, investigadores aíslan la fisostigmina del Calabar y es 50 años después cuando se le agrega un éster de ácido carbámico, denominándolo como carbamato. En 1930 investigadores alemanes reconocieron la toxicidad de los carbamatos y fueron empleados como químicos neurotóxicos en la guerra (Botana López et al., 2002; Gupta, 2006).

A partir de los años 70's, los carbamatos se han empleado de diversas maneras, como herbicidas en la agricultura, ectoparasiticidas en veterinaria, plaguicidas en cuidados forestales e incluso en medicina humana para el tratamiento de algunas enfermedades degenerativas como Alzheimer, miastenia gravis y glaucoma (Botana López et al., 2002; Gupta, 2006), como antimicrobianos, anestésicos locales, anticonvulsivos, antiulcerosos, anticarcinogénicos etc. (Odilón, 1993; Ordaz-Pichardo et al., 2005).

Dentro de los carbamatos utilizados en la actualidad se encuentran el aldicarb, aminocarb, carbaril, carbofuran, carbosulfan, metiocarb entre otros (Gupta, 2006).

En medicina veterinaria los carbamatos empleados para el control de artrópodos son el propoxur y el carbaril. También dentro del grupo de fármacos empleados en veterinaria relacionados a carbamatos se encuentran los benzimidazoles; a pesar de que estos compuestos no pertenecen a esta familia, estos compuestos se ha considerado carbamatos debido a su cercana relación estructural con estos compuestos (Botana López et al., 2002).

## 2.1. Mecanismo de acción

Algunos carbamatos como los N-metil carbamatos tienen una alta afinidad por las esterasas tales como la quimiotripsina, la AChE, la pseudocolinesterasa, carboxilesterasa y otras esterasas no específicas (Córdoba, 2006). La AChE actúa en las sinapsis colinérgicas terminando el impulso nervioso producido por el neurotransmisor acetilcolina. Estos compuestos reaccionan con la AChE de forma análoga a la acetilcolina (figura 3), primero formando un complejo con la enzima (la enzima es carbamilada) con la liberación de un grupo funcional, el paso de recuperación de la enzima se realiza en un tiempo relativamente corto (Sogorb and Vilanova, 2002). La acción inhibitoria en la función de la AChE de los artrópodos, prolonga la excitación nerviosa causada por el neurotransmisor acetilcolina provocando parálisis neuromuscular y la muerte por tetanización (Tan et al., 2011).

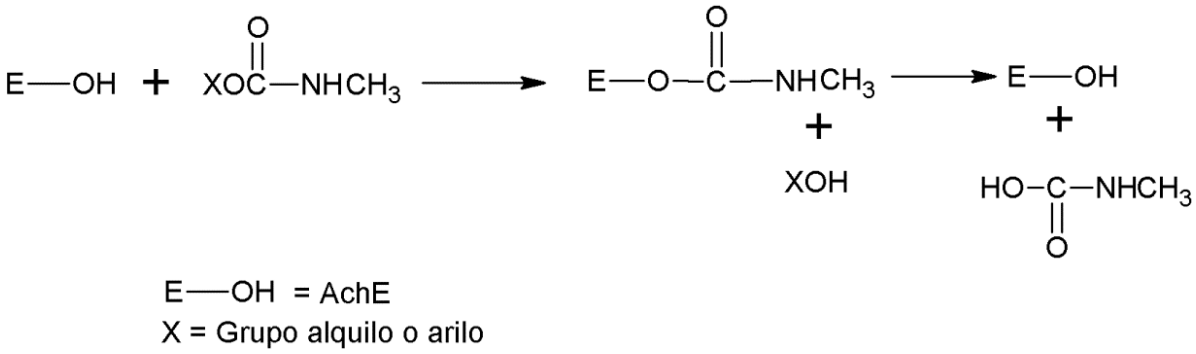


Figura 3. Unión del carbamato a la acetilcolinesterasa, carbamilación (Odilón, 1993)

Por otra parte los carbamatos benzimidazoles han mostrado tener interacción con los centros de organización de los microtúbulos, especialmente con uno de los dímeros que los forman, la β-tubulina, de algunos protozoarios como *Trichomona vaginalis* y *Giardia lamblia,* además sobre helmintos y hongos, provocando efectos en la morfología y sobre el índice mitótico en dichos parásitos (Carvalho and Gadelha, 2007; Chávez et al., 1992; Katiyar et al., 1994); Se ha demostrado que el mecanismo de acción del flubendazol sobre *Toxocara canis* y *Ascaris suum* es el de impedir la polimerización de los microtúbulos en el interior de las células de dichos parásitos, provocando severos daños en su hipodermis e intestinos y evita la formación de gametos (Hanser et al., 2002).

**2.2. Farmacocinética**

La farmacocinética de los carbamatos al igual que la de otros compuestos, se refiere al tiempo que permanece un compuesto químico dentro del organismo desde su absorción hasta su excreción, tomando en cuenta el metabolismo y distribución que este compuesto pueda presentar (Botana López et al., 2002).

*Absorción:* Los carbamatos en general presentan una buena absorción teniendo como principales vías los aerosoles, la piel y el tracto gastrointestinal (Botana López et al., 2002; Gupta, 2006).

*Distribución:* Debido a su alta liposolubilidad**,** Los carbamatos y sus metabolitos difunden hacia todas las partes del cuerpo, dividiéndolo en 7 compartimentos, los cuales son: piel, grasa, tejidos alta perfusión (riñón, bazo), tejidos baja perfusión (hueso, músculo), diafragma, cerebro e hígado (Botana López et al., 2002; Gupta, 2006).

*Metabolismo:* Los carbamatos se hidrolizan por carboxilesterasas, formando α-naftol y acido metil carbámico, el cual se degrada en monometilamina y $CO_2$; el α-naftol se conjuga con sulfatos o glucoronatos. Primero intervienen las enzimas de fase 1, dentro de las cuales se encuentran las enzimas p450, A esterasas

(arilesterasa), B esterasas (carboxilesterasas, butirilcolinesterasas, AChE y la flavino monooxigenasa). La actividad principal de las enzimas de la fase 1 es la de modificar la polaridad de los carbamatos y sus metabolitos, volviéndolos ligeramente hidrosolubles. También intervienen las enzimas de la fase 2, dentro de las que se encuentran la glutatión transferasa, glucuronil transferasa y sulfonil transferasa cuya actividad principal es hidrosolubilizar al carbamato y su metabolito para poder excretarlo (Gupta, 2006; Botana, 2002).

*Excreción:* Los productos derivados del metabolismo de los carbamatos se eliminan por la orina y heces, aunque se ha encontrado también excreción a través de bilis, leche y vías respiratorias (Córdoba, 2006).

### 2.3. Toxicidad de los carbamatos

Las intoxicaciones (no mortales) por carbamatos suelen durar menos de 24 horas. La toxicidad derivada de la inhibición de la enzima AChE por los carbamatos, provoca una acumulación de los niveles de acetilcolina, la cual genera una estimulación de los receptores colinérgicos de forma prolongada y persistente irrumpiendo el equilibrio de la sinapsis. Esta afección en la neurotransmisión genera diversas signologías, las cuales dependerán del sitio en donde se encuentre el daño; zona pre-ganglionar de nervios simpáticos/parasimpáticos, zona post-ganglionar de nervios parasimpáticos, unión neuromuscular esquelética o sinapsis dentro del sistema nervioso central (Gupta, 2006).

Dentro de los principales signos mostrados en una intoxicación por carbamatos se encuentra el denominado síndrome de SLUDGE (por sus siglas en inglés), en el cual se presenta: ptialismo/sialorrea (S), lagrimeo (L), incontinencia urinaria (U), diarrea (D), contracción gastrointestinal (G) y emesis (E); en menor proporción se ha observado bradicardia, broncorrea, taquicardia, tremor muscular, somnolencia, convulsiones y coma (Gupta, 2006).

De acuerdo a su toxicidad oral aguda, en general los carbamatos son de media o baja toxicidad exceptuándose algunos productos como el carbaril, aldicarb, carbofurán, methomyl, lannate, methavin y nudrin que son de toxicidad alta ($DL_{50}$ < 50 mg/kg). El methomyl está clasificado como altamente tóxico; el aldicarb está clasificado como extremadamente tóxico ya que su $DL_{50}$ se encuentra entre 0.3 y 0.9 mg/kg en animales de experimentación (Orme and Kegley, 2003); el propoxur se clasifica como ligeramente tóxico; el carbaril se considera muy tóxico, con una $DL_{50}$ oral en ratas de 12.5 mg/kg, mientras que la toxicidad de los benzimidazoles es baja (PAN pesticide database) como el albendazol que presenta una $DL_{50}$ en ratas de 1320 -2400 mg/kg.

La toxicidad dérmica de los carbamatos es variable, para el carbaril se han reportado valores de $DL_{50}$ por vía dérmica mayores a 2000 mg/kg en ratas, para el carbofuran la $DL_{50}$ dérmica es mayor a 1000 mg/kg en conejos, el propoxur es ligeramente tóxico por vía dérmica con valores de $DL_{50}$ entre 1000 y 2400 mg/kg en ratas, para el bendiocarb la $DL_{50}$ dérmica es de 566 mg/kg en ratas, mientras que para el fenoxycarb se reporta una $DL_{50}$ dérmica mayor a 5000 mg/kg (Baron et al., 1991).

La lipoperoxidación ha sido sugerida como uno de los mecanismos moleculares involucrados en la toxicidad por carbamatos. Se ha reportado un incremento en la lipoperoxidación en hígado de rata por la administración de propoxur (Seth et al., 2000), carbofurán (Brkic et al., 2008) y otros carbamatos (Ozden et al., 2009).

Además se han reportado efectos genotóxicos ocasionados por carbamatos como carbofuran, carbosulfán, aldicarb, propoxur y etil carbamatos (Hubner et al., 1997). El carbofuran indujo inhibición del índice mitótico, aberraciones cromosómicas, formación de micronúcleos y anormalidades espermáticas, de manera dosis dependiente en ratones administrados con 1.9, 3.8, o 5.7 mg/kg

(Chauhan et al., 2000). El carbosulfan, un pesticida que ha sido ampliamente utilizado en la agricultura y es efectivo contra mosquitos resistentes a piretroides (Guillet et al., 2001) se ha reportado como no-mutagénico en cepas de *Salmonella typhimurium* (TA 97, 98, 100 y 102) pero induce aneuploidías en la cepas D61M de *S. cerevisiae*, además se ha reportado que en linfocitos humanos induce disminución en el índice de replicación e índice mitótico de forma dosis-dependiente así como aberraciones cromosómicas (Topaktas et al., 1996). Otro estudio mostró que la exposición aguda a 1.5, 2.5 y 5 mg/kg de carbosulfán en ratones produjo un incremento dosis-dependiente de la frecuencia de aberraciones cromosómicas, eritrocitos policromáticos micronucleados y anormalidades en la cabeza de los espermatozoides (Giri et al., 2002). El propoxur y su derivado nitroso (NO-propoxur) se reportaron como genotóxicos, ya que al ser evaluados en linfocitos humanos *in vitro*, produjeron un incremento en la frecuencia de intercambio de cromátides hermanas y micronúcleos (Gonzalez Cid et al., 1990).

### 2.4. Nuevos carbamatos sintetizados en FES-Cuautitlán, UNAM.

El grupo de investigadores del Laboratorio de Química Medicinal de la Facultad de Estudios Superiores Cuautitlán de la UNAM, diseñó, sintetizó y caracterizó estructuralmente una serie de nuevos derivados del ácido carbámico. Estos se sintetizaron usando aril y alquilaminas con hidruro de sodio y dietilcarbonato de benceno; se purificaron mediante cromatografía en columna y se recristalizaron. Su estructura fue elucidada mediante las técnicas comunes de espectroscopía (Angeles et al., 2000).

A algunos de estos carbamatos se les ha estudiado su actividad sobre diferentes organismos. Actividad antibiótica sobre *Escherichia coli*, *Salmonella typhi*, *Vibrio cholerae*, *Enterobacter aerogenes y Helicobacter pylori* (Bernal, 2000; Reyes-González, 2007).

En cuanto a su actividad antiparasitaria, se evaluó la eficacia de algunos derivados sobre *Hymenolepis nana*, que es un cestodo que se encuentra en el intestino de humanos y ratones, el cual resultó alterado en su morfología principalmente en la estructura y vellosidad de sus proglótidos (Bernabe-Pérez, 2007).

Se ha demostrado la actividad de dos etil-fenil-carbamatos sobre *Giardia intestinalis*, tanto de cepas susceptibles como resistentes a albendazol, además, al combinar los carbamatos con el albendazol se encontró un marcado sinergismo antiprotozoario *in vitro* (Alcántara y Morales, 2005). Se ha demostrado también la actividad antiamibiana de estos productos, encontrando que los carbamatos LQM 996 y el LQM 177 inhiben el crecimiento de *Entamoeba histolytica* (Ordaz-Pichardo et al., 2005).

En cuanto a la actividad antimicótica de estos carbamatos, se encontró que el carbamato LQM 667 presentó una considerable inhibición del crecimiento de *Trichophyton mentagrophytes* y los carbamatos LQM 938, LQM 996 y LQM 919 mostraron actividad sobre *Aspergillus fumigatus* (Reyes-González, 2007).

### 2.4.1. Antecedentes de toxicidad

Para estudiar el comportamiento genotóxico de los compuestos derivados del ácido carbámico sobre los linfocitos humanos, se evaluó el daño al DNA producido por algunos de estos compuestos (LQM 901, LQM 904, LQM 930 y LQM 996) y se compararon con compuestos antiparasitarios (metronidazol, mebendazol y albendazol) por medio del ensayo cometa sin activación metabólica, en dichos estudios se reportó que el daño al DNA producido por los derivados del ácido carbámico fue cercano al control negativo y similar a los antiparasitarios evaluados (Alcántara y Morales, 2005).

En otro estudio se evaluó la mutagenicidad del carbamato LQM 996, por medio del ensayo de Ames, en el que se encontró que concentraciones de 3.12 a 400 µg/ml, con y sin activación metabólica (mezcla S9), no produjeron mutaciones por cambio de lectura en cepas de *Salmonella typhimurium* de las cepas TA97 y TA98, tampoco produjeron mutaciones por sustitución de pares de bases en la cepa TA100, ni mutaciones debidas al daño oxidativo en la cepa TA102 (Ordaz-Pichardo et al., 2005).

Se evaluó la citotoxicidad de algunos de estos carbamatos en cultivos de células CHO, en dicho estudio se reportaron porcentajes de viabilidad del 80 al 90% con concentraciones de carbamatos entre 1.56 y 6.25 µg/ml, en este estudio demostraron que estos carbamatos pueden utilizarse en dosis mayores *in vitro*, que los benzimidazoles en el tratamiento de *Giardia* resistente a benzimidazoles (Jiménez-Cardoso et al., 2004).

# Capítulo 2

## JUSTIFICACIÓN

*Rhipicephalus microplus* es la garrapata más importante en las áreas tropicales y subtropicales de México, causa grandes pérdidas económicas a la ganadería. Durante muchos años, la estrategia más utilizada para el control de garrapatas ha sido el uso de ixodicidas químicos, sin embargo, la alta presión de selección ejercida por el uso exagerado de éstos ha provocado que aparezca el fenómeno de resistencia a los principales ixodicidas comerciales. Lo anterior obliga a desarrollar nuevas alternativas farmacológicas para el control de éstas garrapatas. Entre estas alternativas se encuentra el desarrollo de nuevas moléculas con actividad sobre las garrapatas. Por lo anterior, es necesario desarrollar y evaluar nuevas moléculas de grupos químicos diferentes a las que actualmente se encuentran en productos comerciales. En la primer parte de este trabajo se avaluó el efecto de 17 nuevos carbamatos sintetizados de la FES-Cuautitlán sobre hembras y larvas de *R. microplus* (capítulo 6).

Los resultados del primer estudio mostraron que los huevos producidos por las garrapatas tratadas tenían características morfológicas diferentes a las producidas por garrapatas no tratadas y lo más importante, no eran viables. Lo anterior, nos hizo pensar que el mecanismo de acción de estos nuevos carbamatos es diferente al de los ixodicidas comerciales. En la literatura se ha reportado que el principal mecanismo de acción de otros carbamatos es inhibiendo la AChE en el sistema nervioso de los artrópodos. Por lo que en el capítulo 7 se presentan los resultados de las cinéticas de inhibición de la AChE de larvas de *R. microplus* y posteriormente un estudio hispatológico de los órganos reproductores de hembras repletas tratadas con los carbamatos que tuvieron la mayor efectividad contra *R. microplus* en el primer experimento.

Una vez evaluado el efecto de estos carbamatos, fue necesario evaluar sus posibles efectos adversos sobre mamíferos, por los que se evaluó la toxicidad oral y dérmica aguda (capítulo 8), toxicidad oral subcrónica (capítulo 9) y genotoxicidad (capítulo 10) en ratas, que son el modelo de elección para evaluar estos efectos.

# Capítulo 3

## HIPÓTESIS

I. Los carbamatos de nueva síntesis tienen efecto sobre la mortalidad de larvas, inhibición de la oviposición de hembras repletas y la eclosión de larvas de garrapatas *Rhipicephalus microplus*.

II. Si el efecto observado en la estructura y viabilidad de los huevos producidos por hembras tratadas con los nuevos carbamatos es diferente al reportado para ixodicidas convencionales entonces el mecanismo de acción de estos carbamatos podría ser diferente

III. Como la estructura química de los carbamatos utilizados tiene similitudes con otros fármacos usados en medicina humana y veterinaria, entonces estos son de baja peligrosidad en exposiciones agudas.

IV. Si estos carbamatos presentan una baja toxicidad aguda, sus efectos a largo plazo son leves, lo que los hace de bajo riesgo para mamíferos.

V. Los carbamatos LQM 919 y LQM 996 no son genotóxicos, o su efecto genotóxico, es menor que el provocado por otros fármacos ixodicidas.

# Capítulo 4

## OBJETIVOS

### OBJETIVO GENERAL

Determinar el efecto de los carbamatos de nueva síntesis sobre garrapatas *Rhipicephalus microplus* así como los efectos adversos que pudieran provocar en mamíferos.

### OBJETIVOS PARTICULARES

- Evaluar el efecto de una serie de nuevos carbamatos diseñados y sintetizados en la FES-Cuautitlán de la UNAM sobre larvas y adultas de *R. microplus*
- Evaluar el efecto de los carbamatos LQM 919 y LQM 996 sobre la AChE de *R. microplus* y determinar los cambios estructurales sufridos por los huevos y órganos reproductores de hembras de *R. microplus* tratadas con los carbamatos.
- Determinar los posibles efectos adversos producidos por la exposición oral aguda y dérmica aguda de los carbamatos LQM 919 y LQM 996, en animales de experimentación (ratas).
- Evaluar los efectos adversos producidos por la exposición repetida (90 días) por vía oral (toxicidad subcrónica) de los carbamatos LQM 919 y LQM 996 en animales ratas.
- Evaluar los efectos *in vitro* de LQM 919 y LQM 996 sobre el índice mitótico, cinética de proliferación celular en linfocitos humanos y la inducción *in vivo* de micronúcleos (genotoxicidad).

# Capítulo 5

## TRABAJOS GENERADOS DE ESTA TESIS

La información obtenida del presente estudio generó la escritura de cinco artículos de investigación y la participación en una patente. Los artículos se presentan en los siguientes capítulos, cada uno incluye resumen, introducción, material y métodos, resultados, discusión y referencias. Los artículos se encuentran en diferentes etapas para su publicación en revistas indizadas y son los siguientes:

I. "Effect of new ethyl and methyl carbamates on biological parameters and reproduction of the cattle tick *Rhipicephalus microplus"*
Publicado en "Veterinary Parasitology"

II. "The action of two ethyl carbamates on acetylcholinesterase and reproductive organs of *Rhipicephalus microplus*.
Aceptado para su publicación en "Veterinary Parasitology"

III. "Assesment of acute oral and dermal toxicity of 2 ethyl-carbamates with activity against *Rhipicephalus microplus* in rats*"*.
Enviado a "BioMed Research International".

IV. "Subchronic toxicity study in rats of two new ethyl-carbamates with acaricidal activity".
Enviado a "Food and Chemical Toxicology"

V. "Genotoxicity and cytotoxicity assessment of new ethyl carbamates with acaricidal activity".
Enviado a "Environmental and Molecular Mutagenesis".

VI. Patente: "Uso de derivados del ácido carbámico como antiparasitarios" con registro ante el Instituto Mexicano de la Propiedad Industrial NO. MX/E/2011/061614 (Anexo 1).

# Capítulo 6

## EFFECT OF NEW ETHYL AND METHYL CARBAMATES ON BIOLOGICAL PARAMETERS AND REPRODUCTION OF THE CATTLE TICK *Rhipicephalus microplus*

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# Effect of new ethyl and methyl carbamates on biological parameters and reproduction of the cattle tick *Rhipicephalus microplus*

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ABSTRACT

The effect of carbamates on engorged female *Rhipicephalus microplus* ticks and larvae was evaluated using the adult immersion test (AIT) and the larval packet test (LPT), respectively. Seventeen synthetic carbamates different from current commercial acaricides were synthesised at the National Autonomous University of Mexico. None of the carbamates had an effect on the percentage of females laying eggs. Six of the compounds inhibited egg laying up to 65.4% and inhibited egg hatching by up to 100% ($p < 0.05$). Compared to untreated females, eggs produced by treated females had a dark, dry, opaque appearance and were less adherent. Carbamates LQM 934 and LQM 938 had an effect on larval mortality ($p < 0.05$). Carbamate LQM 934 showed lethal concentrations (LC) of $LC_{90} = 0.76\%$ and $LC_{99} = 0.87\%$, while LQM 938 showed concentrations of $LC_{90} = 0.267\%$ and $LC_{99} = 0.305\%$. The compounds were distributed into three classes of acaricidal activity using the AIT or the LPT. These three classes were as follows: (1) compounds having no apparent effect; (2) compounds that inhibit egg laying and embryo development or (3) compounds that exhibit acaricidal activity to larval ticks.



## 1. Introduction

*Rhipicephalus microplus* is the most important cattle tick in tropical and subtropical areas of Mexico as well as other parts of the world. Infestations of cattle produce anaemia, a decrease in growth and reproductive parameters, a reduction of meat and milk production, a reduction in the quality of pelts and increased transmission of diseases, such as babesiosis and anaplasmosis (Solorio-Rivera and Rodriguez-Vivas, 1997; Oliveira et al., 2005; Jonsson et al., 2008). For many years, the most common strategy for *R. microplus* control has been the use of chemical acaricides, although the high selection pressure exerted by the use of current commercial acaricides has led to acaricide resistance (Soberanes-Céspedes et al., 2002; Foil et al., 2004; George et al., 2004). These facts mandate the development of new pharmacological alternatives for tick control. In this context, the synthesis of new chemicals is an attractive strategy for overcoming acaricide resistance.

Carbamates are a large group of carbamic ester compounds. In general, they are crystals that are stable between 10 and 40 °C. Although some carbamates are currently used as antiparasitics and insecticides, there are no carbamates with specific efficacy against ticks (Gupta, 2006). The mechanisms of action of these compounds are variable:

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some carbamates, such as Carbaryl, Carbofuran, Methomyl and Propoxur, act similarly to organophosphates by inhibiting acetylcholinesterase; others, such as benzimidazoles, prevent the polymerisation of tubulin in protozoa and nematodes (Chávez et al., 1992; Katiyar et al., 1994; Hanser et al., 2003; Sultatos, 2006; Carvalho and Gadelha, 2007). This study evaluated the effect of a series of new carbamates on *R. microplus* engorged females and tick larvae; these carbamates were synthesised at the National Autonomous University of Mexico (Angeles et al., 2000). These synthetic carbamates belong to a different chemical group than any of the current commercial acaricides.

## 2. Materials and methods

### 2.1. R. microplus tick strain

The Media Joya strain of *R. microplus*, which is susceptible to conventional acaricides used in Mexico (organophosphates, pyrethroids and amidines), was used. This strain was collected from a ranch in the state of Jalisco in Mexico and has been bred at the Centro Nacional de Investigacion Disciplinaria en Parasitologia Veterinaria Instituto Nacional de Investigaciones Forestales y Agropecuarias since 2001; currently, it is used as a susceptible reference strain for Latin America (Martínez et al., 2008; Aguilar-Tipacamú et al., 2008). The strain was donated by the National Animal Health Verification Services Center of the State Animal Health Office (SAGARPA) of Mexico and has been maintained on Aberdeen Angus cattle. This study was approved by the Internal Committee for Care of Experimental Animals of the Postgraduate Program of Animal Production and Health (UNAM, Mexico).

### 2.2. Experimental design

The effects of 17 synthetic carbamates on engorged *R. microplus* females and larvae were evaluated. To identify the carbamates with activity against engorged *R. microplus* females, the adult immersion test (AIT) was used with a 1 mg/ml concentration. Carbamates that significantly increased the percent inhibition of egg laying and/or decreased the percentage of egg hatching in the bioassay before were again evaluated using the AIT with the following concentrations: 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml, 0.03125 mg/ml and 0 mg/ml (negative control). Egg hatching inhibitory concentrations (EHIC) were estimated by Probit analysis.

The effects of the 17 carbamates on *R. microplus* larvae were evaluated using the larval packet test (LPT). Eight serial dilutions were prepared and following concentrations were used: 1%, 0.25%, 0.0625%, 0.0156%, 0.0039%, 0.0009%, 0.0002% and 0% (negative control). Carbamates that produced 100% larval mortality at the 1% and 0.25% concentrations were evaluated using a second series of concentrations: 1%, 0.91%, 0.82%, 0.73%, 0.64%, 0.55%, 0.25%, 0.235%, 0.22%, 0.205%, 0.19%, 0.175% and 0% (negative control). Probit analysis was used to estimate the 90% and 99% lethal concentrations ($LC_{90}$ and $LC_{99}$, respectively).

### 2.3. Evaluated carbamates

The carbamates used in this study were designed and synthesised at the National Autonomous University of Mexico, using a benzimidazole molecule as the structural base. They were synthesised by reacting aryl- and alkylamines with sodium hydride and benzene diethylcarbonate and were then purified using column chromatography; next, the products were recrystallised. They were structurally characterised by interpretation of their infrared spectra, hydrogen and carbon-13 nuclear magnetic resonance and mass spectrometry (Angeles et al., 2000).

The tested carbamates were insoluble in water; therefore, for the AIT, the carbamates were dissolved in dimethylsulphoxide (DMSO), and then this solution was dissolved in distilled water. The final concentration of DMSO in all assays was 4%. The chemical structure, nomenclature, molecular weight and identification code of the carbamates tested can be found in Table 1.

### 2.4. Adult immersion test

The percentage of females laying eggs, the percent inhibition of egg laying and the percentage of egg hatching were determined using the AIT as described by Drummond et al. (1976) and the modified methods published by Sardá-Ribeiro et al. (2008). Each concentration was evaluated in triplicate using groups of 10 engorged female ticks collected the same day. Engorged female ticks were weighed and immersed for 30 min in the appropriate concentration of carbamate (25 ml) in a Becker flask, which was gently agitated at room temperature. Ticks from each group were recovered from the solutions, dried and dorsally attached to a Petri dish with masking tape. The Petri dishes were incubated at 28 °C with 70–80% relative humidity. After 14 days, the number of females in each dish laying eggs was recorded and the eggs were collected, weighed and observed. The percentage inhibition of egg laying was calculated as follows:

$$\text{Index of egg laying (IE)} = \frac{\text{weight of eggs laid (g)}}{\text{weight of females (g)}}$$

$$\%\,\text{inhibition of egg laying} = \frac{\text{IE negative control group} - \text{IE treated group}}{\text{IE negative control group}} \times 100\%$$

The collected eggs were placed in a 15 ml glass vial and incubated at 28 °C with 70–80% relative humidity. After 30 days, the percentage of hatched and unhatched eggs was recorded and the $EHIC_{50}$ and $EHIC_{99}$ were calculated by Probit analysis. Engorged female ticks immersed in the commercial products Asuntol® (Coumaphos 200 ppm), Bovitraz® (Amitraz 250 ppm) and Bayticol® (Flumethrin 30 ppm) from Bayer, Mexico were used as positive controls. Engorged female ticks that had been immersed in 4% DMSO were used as the negative control group for calculation of the percent inhibition of egg laying.

**Table 1**
Chemical structures and molecular weights of the evaluated carbamates.

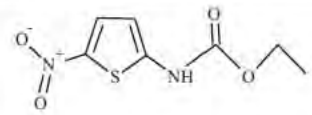
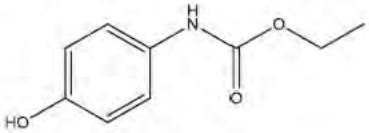
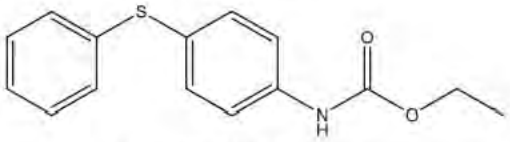
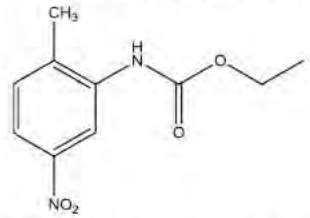
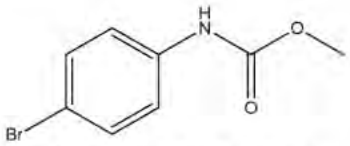
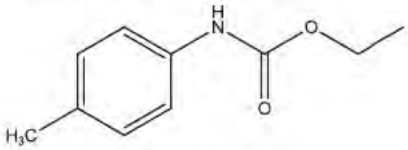
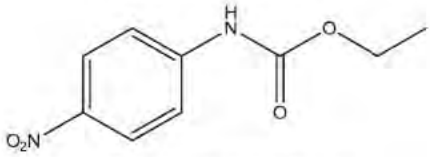
| Carbamate identification code | Chemical structure | Molecular weight (g/mol) |
|---|---|---|
| LQM 006 |  ethyl (5-nitrothiophen-2-yl)carbamate | 216.21 |
| LQM 181 |  ethyl (4-hydroxyphenyl)carbamate | 181 |
| LQM 667 |  ethyl (4-(phenylthio)phenyl)carbamate | 273 |
| LQM 903 |  ethyl (2-methyl-5-nitrophenyl)carbamate | 224.21 |
| LQM 904 |  methyl (4-bromophenyl)carbamate | 230 |
| LQM 906 |  ethyl *p*-tolylcarbamate | 179 |
| LQM 914 |  ethyl (4-nitrophenyl)carbamate | 210 |

Table 1 (*Continued*)

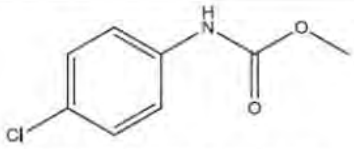
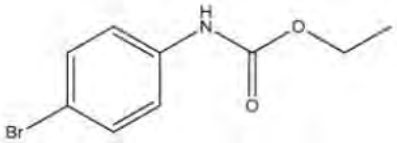
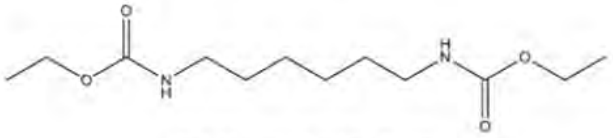
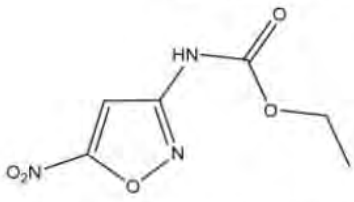
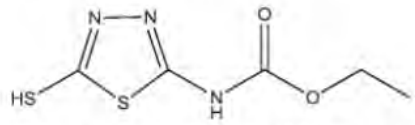
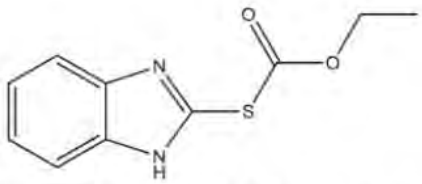
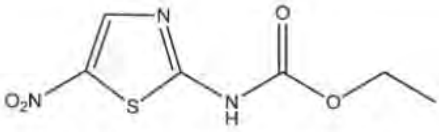
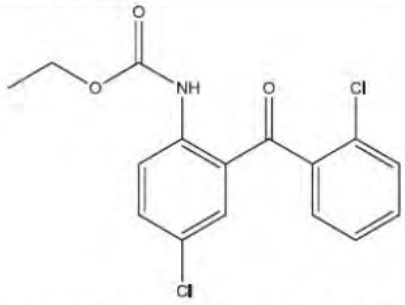
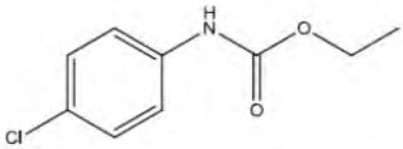
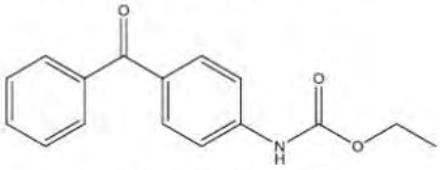
| Carbamate identification code | Chemical structure | Molecular weight (g/mol) |
|---|---|---|
| LQM 917 |  methyl (4-chlorophenyl)carbamate | 185.5 |
| LQM 919 |  ethyl (4-bromophenyl)carbamate | 244 |
| LQM 932 |  diethyl hexane-1,6-diyldicarbamate | 260.33 |
| LQM 934 |  ethyl (5-nitroisoxazol-3-yl)carbamate | 170 |
| LQM 935 |  ethyl (5-mercapto-1,3,4-thiadiazol-2-yl)carbamate | 205.26 |
| LQM 936 |  *S*-1*H*-benzo[*d*]imidazol-2-yl *O*-ethyl carbonothioate | 222 |
| LQM 938 |  ethyl (5-nitrothiazol-2-yl)carbamate | 217 |

Table 1 (*Continued*)

| Carbamate identification code | Chemical structure | Molecular weight (g/mol) |
|---|---|---|
| LQM 939 |  ethyl (4-chloro-2-(2-chlorobenzoyl)phenyl)carbamate | 338 |
| LQM 996 |  ethyl (4-chlorophenyl)carbamate | 199.63 |
| LQM 997 |  ethyl (4-benzoylphenyl)carbamate | 269.29 |

### 2.5. Larval packet test

Larval mortality was estimated using the LPT as described by Stone and Haydock (1962). Initially, a 1% stock solution of each carbamate was prepared in a mixture containing two parts trichloroethylene and one part commercial olive oil. This stock solution was used to prepare serial dilutions (see above) and a piece of 750 mm × 850 mm filter paper (Whatman No. 1) was impregnated with 0.67 ml of each of the solutions. The papers were dried for 1 h at room temperature to allow trichloroethylene evaporation. After drying, the filter papers were folded and sealed with a metal clip to form packets. Each concentration was evaluated in triplicate using packets containing approximately 100 14-day-old larvae. The packets were sealed and incubated at 28 °C with 70–80% relative humidity. After 24 h, larval mortality was determined by counting the total dead and alive individuals. Paralysed larvae were considered dead. For each carbamate, the $LC_{90}$ and $LC_{99}$ were calculated using Probit analysis. Packets impregnated with trichloroethylene and olive oil (2:1) were used as negative controls.

### 2.6. Statistical analysis

The larval mortality data, the percent inhibition of egg laying and the egg hatching percentage were analysed by one way ANOVA and Tukey's test at a 95% confidence level using the Statistica 7 software from StatSoft®. This analysis was carried out to determine which groups showed a statistically significant effect when compared to the negative control groups.

The $LC_{90}$, $LC_{99}$, $EHIC_{50}$ and $EHIC_{99}$ were estimated by Probit analysis (dose-response) using the Polo-Plus software (Le-Ora-Software, 2004) with a 95% confidence limit (CL).

## 3. Results

### 3.1. Adult immersion test

Immersion of engorged female *R. microplus* ticks in a 1 mg/ml solution of each carbamate had no statistically significant effect ($p > 0.05$) on the percentage of females laying eggs in comparison with the negative control. Mortality of the treated engorged females was not observed. Nevertheless, carbamates LQM 904, LQM 906, LQM 914, LQM 917, LQM 919 and LQM 996 at this same concentration (1 mg/ml) increased the percent inhibition of egg laying and decreased the percentage of egg hatching ($p < 0.05$) compared with the negative control. To calculate the EHIC, these six carbamates were tested at several concentrations. The egg laying inhibition and egg hatching percentage for each product are presented in Tables 2 and 3. Most of the engorged female ticks used in the positive control groups died and therefore did not produce eggs; this group were

**Table 2**
Percentage inhibition of egg-laying of *Rhipicephalus microplus* engorged females immersed in several concentrations of various synthetic carbamates.

| Concentration | Carbamate identification code | | | | | |
|---|---|---|---|---|---|---|
| | LQM 904 | LQM 906 | LQM 914 | LQM 917 | LQM 919 | LQM 996 |
| 1 mg/ml | 47.0 (±6.5)* | 36.1 (±5.9)* | 51.1 (±2.7)* | 59.8 (±5.0)* | 54.7 (±0.6)* | 62.7 (±2.3)* |
| 0.5 mg/ml | 53.4 (±8.2)* | 21.3 (±5.6)* | 42.8 (±5.2)* | 68.8 (±4.4)* | 52.7 (±7.1)* | 55.1 (±3.3)* |
| 0.25 mg/ml | 43.9 (±3.5)* | 13.3 (±4.5) | 34.7 (±1.9)* | 27.7 (±3.9)* | 35.9 (±9.8)* | 37.9 (±3.4)* |
| 0.125 mg/ml | 4.8 (±8.7) | 10.0 (±3.6) | 1.4 (±3.6) | 15.7 (±2.6) | 18.6 (±8.1) | 20.0 (±3.6)* |
| 0.0625 mg/ml | −6.8 (±4.4) | 13.0 (±2.6) | −11.3 (±6.2) | 7.9 (±8.4) | 9.5 (±6.9) | 16.0 (±3.2) |
| 0.03125 mg/ml | −3.5 (±10.5) | 4.4 (±7.9) | −3.1 (±5.9) | 11.1 (±5.6) | −3.8 (±5.7) | −2.6 (±3.3) |
| Control ($H_2O$) | −5.3 (±1.9) | −1.8 (±2.9) | −1.7 (±0.3) | 2.7 (±3.9) | −4.0 (±1.1) | −0.70 (±1.9) |
| Control ($H_2O$ + DMSO) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

only used as test control, and were not used to estimate any parameter.

### 3.2. Egg hatching inhibitory concentration

The EHIC values are shown in Table 4. The six carbamates that were inhibitors of egg laying and egg hatching affected the physical characteristics of the eggs that were laid by the treated ticks. Eggs produced by the treated engorged female ticks had a dark, dry, opaque appearance relative to those produced by untreated females. It was also observed that adherence among eggs decreased (Fig. 1). Because the efficacy data for LQM 914 did not fit the Probit analysis model, it was not possible to calculate its EHIC values; however the minimal concentration with an effect on the egg hatching percentage was 0.125 mg/ml (Table 3).

### 3.3. Larval packet test

The carbamates LQM 934 and LQM 938 in the first series of assays had a statistically significant effect ($p < 0.05$) on larval mortality compared with the negative control (between 0 and 1% larval mortality). The carbamate LQM 934 caused 100% larval mortality at the 1% concentration, while carbamate LQM 938 caused 100% larvae mortality at both 1% and 0.25% concentrations. With the data obtained from the second series of assays, carbamate LQM 934 had an $LC_{90}$ = 0.760% (CL = 0.738–0.789) and an $LC_{99}$ = 0.870% (CL = 0.872–0.924), while LQM 938 had an $LC_{90}$ = 0.267% (CL = 0.256–0.284) and an $LC_{99}$ = 0.305% (CL = 0.286–0.337).

## 4. Discussion

Carbamates are compounds that have a wide variety of chemical properties and different applications. They have primarily been used as insecticides, herbicides, nematicides and fungicides (Gupta, 2006). Due to variable chemical properties, the effects of known carbamates cannot be extrapolated to this study. More studies are needed to be done in order to determine the mechanisms of action.

The carbamates evaluated in this study were tested using the AIT (Drummond et al., 1976), which has been widely applied for evaluation of *R. microplus* tick susceptibility to acaricides (de Freitas-Fernandes and de Paula Souza Freitas, 2007; Pereira and Famadas, 2006; Sardá-Ribeiro et al., 2008; Alonso-Díaz et al., 2007). Some modifications were made to the AIT technique; for instance, we used DMSO to dissolve the carbamates because they are not water-soluble. The effect of DMSO on ticks has not been previously documented. Therefore, an additional control group treated with DMSO and water was used. No differences were found in any of the evaluated parameters between this group and a group treated with water only. As a result, we concluded that the effects observed in the study were due to the carbamates being tested and not due to the DMSO.

Carbamates that had an effect on the egg mass reduction and egg hatching (LQM 904, LQM 906, LQM 914, LQM 917, LQM 919 and LQM 996) did not have an effect on the number of females laying eggs, but they did have an effect on the weight of the egg masses produced. Eggs laid by treated females displayed different physical characteristics (opaque, dark, dry and disaggregated eggs) from the eggs produced by untreated females. In addition, the eggs of these carbamate-treated adult ticks were not viable because no larvae emerged from them after 30 days in optimal conditions. We thus determined that these carbamates interfere with the biological life cycle of ticks.

**Table 3**
Percentage of egg-hatching produced by engorged females of *Rhipicephalus microplus* immersed in several concentrations of various synthetic carbamates.

| Concentration | Carbamate identification code | | | | | |
|---|---|---|---|---|---|---|
| | LQM 904 | LQM 906 | LQM 914 | LQM 917 | LQM 919 | LQM 996 |
| 1 mg/ml | 8.1 (±1.4)* | 0.0 (±0.0)* | 0.0 (±0.0)* | 0.0 (±0.0)* | 0.0 (±0.00)* | 0.0 (±0.0)* |
| 0.5 mg/ml | 55.60 (±4.2) | 1.2 (±1.28)* | 0.0 (±0.0)* | 0.0 (±0.0)* | 1.2 (±1.2)* | 0.0 (±0.0)* |
| 0.25 mg/ml | 58.9 (±7.31) | 8.6 (±4.6)* | 0.0 (±0.00)* | 18.9 (±4.8)* | 8.6 (±4.6)* | 1.0 (±0.6)* |
| 0.125 mg/ml | 57.4 (±3.8) | 39.4 (±3.40) | 47.0 (±9.0)* | 23.2 (±2.6)* | 39.4 (±3.4) | 35.2 (±1.1)* |
| 0.0625 mg/ml | 65.9 (±5.08) | 53.7 (±7.2) | 68.7 (±4.3) | 65.4 (±10.1) | 53.7 (±7.2) | 67.0 (±2.5) |
| 0.03125 mg/ml | 64.7 (±1.4) | 65.1 (±4.1) | 67.4 (±5.0) | 70.8 (±0.7) | 65.1 (±4.1) | 68.7 (±2.1) |
| Control ($H_2O$) | 67.7 (±1.3) | 65.01 (±1.0) | 69.5 (±7.8) | 65.0 (±2.1) | 65.0 (±1.0) | 71.4 (±2.9) |
| Control ($H_2O$ + DMSO) | 68.3 (±5.2) | 66.1 (±3.6) | 74.8 (±4.2) | 76.0 (±2.5) | 66.1 (±3.6) | 69.9 (±6.7) |

**Table 4**
Egg hatching inhibitory concentrations of the carbamates with an effect on *Rhipicephalus microplus* egg hatching.

| Carbamate identification code | EIIC$_{50}$ (mg/ml) | CI 95% | EIIC$_{99}$ (mg/ml) | CI 95% |
|---|---|---|---|---|
| LQM 904 | 0.661 | 0.515–0.755 | 1.493 | 1.218–2.419 |
| LQM 905 | 0.133 | 0.104–0.158 | 0.537 | 0.401–0.911 |
| LQM 917 | 0.137 | 0.100–0.171 | 0.625 | 0.438–1.222 |
| LQM 919 | 0.097 | 0.083–0.112 | 0.687 | 0.526–0.994 |
| LQM 996 | 0.121 | 0.114–0.128 | 0.279 | 0.249–0.326 |

The effective concentrations of some acaricides, as determined by AIT, have been reported for susceptible strains of *R. microplus*; these include Coumaphos (200 ppm), Chlorfenvinphos (300 ppm), Chlorpyriphos (300 ppm), Ethion (560 ppm), Flumethrin (40 ppm), Cypermethrin (150 ppm), Amitraz (150 ppm) and Deltamethrin (20 ppm) (Foil et al., 2004). In this study, the products that had the lowest effective concentrations (EHIC$_{99}$) were LQM 906 (537 ppm), LQM 914 (250 ppm) and LQM 996 (279 ppm). The efficacy of the carbamates was not measured; as such, it is not possible to state that the carbamates tested have a greater or lower efficacy than commercially available acaricides. However, we can conclude that the effective concentrations found in this study are within the ranges reported for the products currently used for tick control.

The products that were effective on engorged female ticks did not show larvicide activity because, in the LPT no death of treated larvae could be observed at any of the product concentrations tested.

LPT has been proposed as a conventional method for testing susceptibility to acaricides in *R. microplus* (FAO, 2004), and several researchers have used it to evaluate new products (Sabatini et al., 2001; Sardá-Ribeiro et al., 2008; de Freitas-Fernandes and de Paula Souza Freitas, 2007). Of the 17 carbamates evaluated using LPT, only LQM 934 (LC$_{99}$ = 0.870%) and LQM 938 (LC$_{99}$ = 0.305%) had an effect on larval mortality. The LC of these carbamates were within the effectiveness range of compounds such as Coumaphos (LC$_{99}$ = 0.155%) and Permethrin (LC$_{99}$ = 0.214%), which are widely used as commercial acaricides (Davey and George, 1998).

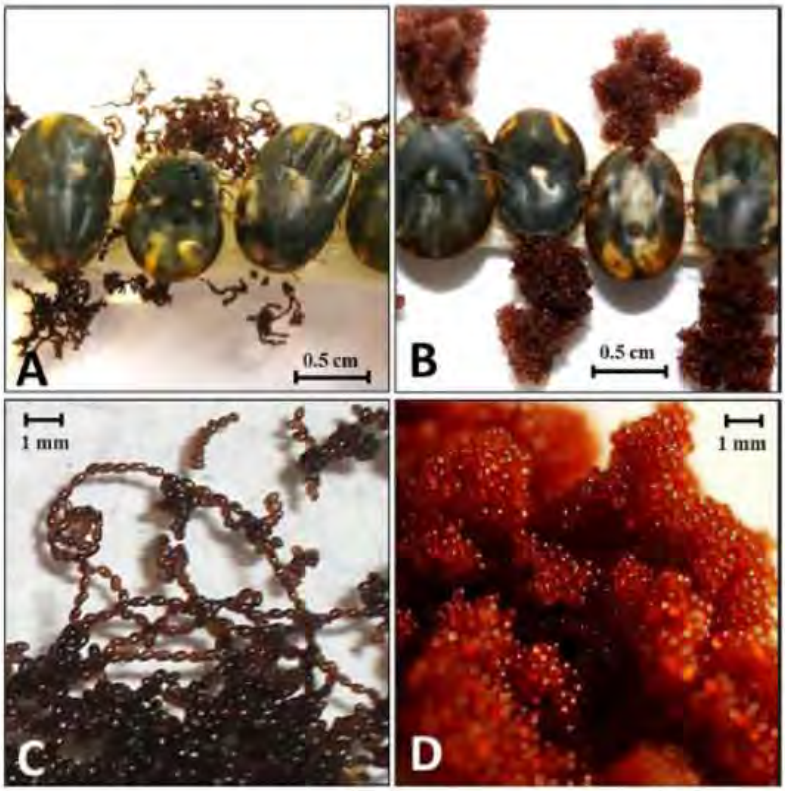



**Fig. 1.** (A) Female *Rhipicephalus microplus* ticks were immersed in the synthetic carbamate LQM 919 (1 mg/ml), (B) female *R. microplus* ticks were immersed in water and dimethylsulphoxide (control group), (C) eggs produced by female *R. microplus* ticks were immersed in the synthetic carbamate LQM 919, (D) eggs produced by female *R. microplus* ticks were immersed in water and dimethylsulphoxide (control group). Similar effects were observed in the synthetic carbamates LQM 904, LQM 906, LQM 914, LQM 917 and LQM 996.

The criteria for considering larvae as dead were either immobility or the incapacity to displace itself with uncontrolled movement (paralysis). When larvae treated with LQM 934 or LQM 938 were observed under the microscope, we noted that most of them were paralysed.

The carbamates that had an effect on larvae did not cause death in engorged female ticks, nor did they have an effect on egg laying when evaluated with AIT. Nevertheless, it is not possible to determine *in vitro* if a pharmaceutical product can produce paralysis of adult ticks, resulting in release from the host. To address this, it is necessary to carry out *in vivo* tests to evaluate the effects of these carbamates on the various stages of the tick lifecycle (larvae, nymph and adult).

Some reports indicate that there is cross-resistance between organophosphates and carbamates (Kunz and Kemp, 1994; Chevillon et al., 2007), which could also cause resistance to the carbamates used in this study. Further assays are required to evaluate the activity of carbamates LQM 934 and LQM 938 in strains of ticks that are resistant to organophosphates.

Carbamate toxicity is highly variable. In general, they have medium to low toxicity, but some of them, such as carbaryl and aldicarb, are highly toxic (U.S. Environmental Protection Agency, 1998). In contrast, the benzimidazole type has low toxicity (Kegley et al., 2010). The carbamates used in this work have only recently been synthesised; therefore, it is unknown if they produce adverse effects on mammals. The authors have determined in previous studies that acute oral and dermal toxicity of these compounds in rats is low (unpublished data). Nevertheless, these carbamates are currently under genotoxicity, cytotoxicity and subchronic toxicity testing prior to efficacy tests against ticks *in vivo*.

The results from the *in vitro* evaluation of these new compounds are promising. Nevertheless, it is important to evaluate the efficacy of these products on tick strains showing double and triple resistance to various conventional acaricides because the primary objective of the development of new products is to control resistant strains of ticks. Future studies should assess the potential of such products in tick control.

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## References

Aguilar-Tipacamú, G., Miller, R.J., Hernández-Ortiz, R., Rodríguez-Vivas, R.I., Vásquez-Peláez, C., García-Vázquez, Z., Olvera-Valencia, F., Rosario-Cruz, R., 2008. Inheritance of pyrethroid resistance and a sodium channel gene mutation in the cattle tick *Boophilus microplus*. Parasitol. Res. 103, 633–639.

Alonso-Díaz, M.A., García, L., Galindo-Velasco, E., Lezama-Gutiérrez, R., Angel-Sahagún, C.A., Rodríguez-Vivas, R.I., Fragoso-Sánchez, H., 2007. Evaluation of *Metarhizium anisopliae* (Hyphomycetes) for the control of *Boophilus microplus* (Acari: Ixodidae) on naturally infested cattle in the Mexican tropics. Vet. Parasitol. 147, 336–340.

Angeles, E., Martínez, P., Keller, J., Martínez, R., Rubio, M., Ramírez, G., Castillo, R., López-Castañares, R., Jiménez, E., 2000. Ethyl and methyl phenyl carbamates as antihelminthic agents: theoretical study for predicting their biological activity by PM3. J. Mol. Struct: Theochem. 504, 141–170.

Carvalho, K.P., Gadelha, A.P., 2007. Effects of three benzimidazoles on growth, general morphology and ultrastructure of *Tritrichomonas foetus*. FEMS Microbiol. Lett. 275, 292–300.

Chávez, B., Cedillo-Rivera, R., Martínez-Palomo, A., 1992. *Giardia lamblia*: ultrastructural study of the in vitro effect of benzimidazoles. J. Protozool. 39, 510–515.

Chevillon, C., Ducornez, S., de Meeûs, T., Koffi, B.B., Gaïa, H., Delathière, J., Barré, N., 2007. Accumulation of acaricide resistance mechanisms in *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) populations from New Caledonia Island. Vet. Parasitol. 147, 276–288.

Davey, R.B., George, J.E., 1998. In vitro and in vivo evaluations of a strain of *Boophilus microplus* (Acari: Ixodidae) selected for resistance to permethrin. J. Med. Entomol. 35, 1013–1019.

de Freitas-Fernandes, F., de Paula Souza Freitas, E., 2007. Acaricidal activity of an oleoresinous extract from *Copaifera reticulate* (Leguminosae: Caesalpinioideae) against larvae of the southern cattle tick, *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae). Vet. Parasitol. 147, 150–154.

Drummond, R.O., Ernst, S.E., Treviño, J.L., Gladney, W.J., Graham, O.H., 1976. Test of acaricides for control of *Boophilus annulatus* and *Boophilus microplus*. J. Econ. Entomol. 69, 37–40.

FAO Working Group on Parasite Resistance, 2004. Resistance Management and Integrated Parasite Control in Ruminants: Guidelines. http://www.fao.org/ag/aga.html

Foil, L.D., Coleman, P., Eisler, M., Fragoso-Sanchez, H., Garcia-Vazquez, Z., Guerrero, F.D., Jonsson, N.N., Langstaff, I.G., Li, A.Y., Machila, N., 2004. Factors that influence the prevalence of acaricide resistance and tick-borne diseases. Vet. Parasitol. 125, 163–181.

George, J.E., Pound, J.M., Davey, R.B., 2004. Chemical control of ticks on cattle and the resistance of these parasites to acaricides. Parasitology 129, 353–366.

Gupta, R.C., 2006. Classification and uses of organophosphates and carbamates. In: Gupta, R.C. (Ed.), Toxicology of Organophosphate & Carbamate Compounds. Elsevier Academic Press, San Diego, pp. 5–24.

Hanser, E., Mehlhorn, H., Hoeben, D., Vlaminck, K., 2003. In vitro studies on the effects of flubendazole against *Toxocara canis* and *Ascaris suum*. Parasitol. Res. 89, 63–74.

Jonsson, N.N., Bock, R.E., Jorgensen, W.K., 2008. Productivity and health effects of anaplasmosis and babesiosis on *Bos indicus* cattle and their crosses, and the effects of differing intensity of tick control in Australia. Vet. Parasitol. 155, 1–9.

Katiyar, S.K., Gordon, V.R., McLaughlin, G.L., Edlind, T.D., 1994. Antiprotozoal activities of benzimidazoles and correlations with beta-tubulin sequence. Antimicrobial A. Chem. 38, 2086–2090.

Kegley, S.E., Hill, B.R., Orme, S., Choi, A.H., 2010. PAN Pesticide Database. Pesticide Action Network, North America San Francisco, CA http://www.pesticideinfo.org

Kunz, S.E., Kemp, D.H., 1994. Insecticides and acaricides: resistance and environmental impact. Rev. Sci. Tech. Off. Int. Epiz. 13, 1249–1286.

Le-Ora, Software, 2004. A user's Guide to Probit or Logit Analysis. Le Ora Software, Berkeley, CA, USA.

Martínez, I.F., Ortiz, N.A., Osorio, M.J., 2008. Comportamiento toxicológico a organofosforados y piretroides de *Boophilus microplus* cepa susceptible (CENAPA) del año 2002 al 2005, posterior a su caracterización. In: Memorias del VI Seminario Internacional de Parasitología Animal Boca del Río, Ver, México.

Oliveira, M., Oliveira-Sequeira, T., Araujo, J., Amarante, A., Oliveira, H., 2005. *Babesia* spp. infection in *Boophilus microplus* engorged females and eggs in Sao Paulo State. Brazil. Vet. Parasitol. 130, 61–67.

Pereira, J.R., Famadas, K.M., 2006. The efficiency of extracts of *Dahlstedtia pentaphylla* (Leguminosae, Papilionoidae, Millettiedae) on *Boophilus microplus* (Canestrini, 1887) in artificially infested bovines. Vet. Parasitol. 142, 192–195.

Sabatini, G.A., Kemp, D.H., Hughes, S., Nari, A., Hansen, J., 2001. Test to determine LC50 and discriminating doses for macrocyclic lactones against the cattle tick, *Boophilus microplus*. Vet. Parasitol. 95, 53–62.

Sarlá Ribeiro, V.L., Avancini, C., Gonçalves, K., Toigo, E., Von Poser, G., 2008. Acaricidal activity of *Calea serrata* (Asteraceae) on *Boophilus microplus* and *Rhipicephalus sanguineus*. Vet. Parasitol. 151, 351–354.

Sobrados Céspedes, N., Santamaría Vargas, M., Fragoso Sánchez, H., García Vázquez, Z., 2002. Primer caso de resistencia al amitraz en la garrapata del ganado *Boophilus microplus* en México. Tec. Pecu. Mex. 40, 81–93.

Solorio Rivera, J., Rodríguez Vivas, R., 1997. Epidemiología de la babesiosis bovina, I. componentes epidemiológicos. Rev. Biomed. 8, 37–47.

Stone, B., Haydock, K., 1962. A method for measuring the acaricide susceptibility of the cattle tick *Boophilus microplus* (Can.). Bull. Entomol. Res. 53, 563–578.

Sultatos, L.G., 2006. Interactions of organophosphorus and carbamate compounds with cholinesterases. In: Gupta, R.C. (Ed.), Toxicology of Organophosphate & Carbamate Compounds. Elsevier Academic Press, San Diego, pp. 209–218.

U.S. Environmental Protection Agency, 1988. Twenty third report of the interagency testing committee to the administrator; receipt of report and request for comments regarding priority list of chemicals. Fed. Reg. 53, 46244–46272.

# Capítulo 7

## THE ACTION OF TWO ETHYL CARBAMATES ON ACETYLCHOLINESTERASE AND REPRODUCTIVE ORGANS OF *Rhipicephalus microplus*

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**ABSTRACT**

The effects produced by the new synthetic carbamates ethyl-(4-bromophenyl) carbamate and ethyl-(4-chlorophenyl) carbamate on the acetylcholinesterase (AChE) activity, egg structure and reproductive organs of two *Rhipicephalus microplus* strains were evaluated. Inhibition kinetic parameters showed that the studied carbamates are weak inhibitors and have a low affinity for *R. microplus* AChE. Histologically, in oocytes from carbamate-treated engorged female ticks, a loss of shape, cytoplasmic vacuoles, decreased chorion deposition, alterations in cytoplasmic granularity and irregular membranes were observed. In oocyte germinal vesicles, a loss of shape, nucleolar fragmentation and membrane alterations with degenerative signs were observed. The ovarian epithelium was vacuolated, flattened, eroded and contained pyknotic nuclei. These alterations were observed from the first day and persisted and increased in severity until day 7 post-treatment. The ovaries from carbamate-treated ticks had fewer stage IV-V oocytes and more stage I-II oocytes. Additionally, eggs produced by the treated ticks had a modified appearance, decreased size, a reduced superficial waxy layer and a loss of viability. The results of this study show that the effects of carbamates on *R. microplus* were independent of AChE inhibition and show that the morphological alterations in the reproductive organs were due to carbamate actions on the vitellogenesis and viability of the ovarian cells.

Keywords: *Rhipicephalus microplus*; carbamates; acetylcholinesterase; oocytes; histology; reproductive organs; ovary, ticks

## INTRODUCTION

The indiscriminate use of ixodicides has produced the emergence of drug-resistant tick strains, leading to increased difficulties in control in some regions (Soberanes-Céspedes et al., 2002). The development of new ixodicides is a strategy with which to counter such resistance. Our group has demonstrated that new synthetic carbamates, such as ethyl-(4-bromophenyl) carbamate (LQM 919) and ethyl-(4-chlorophenyl) carbamate (LQM 996), can negatively affect *R. microplus* biological parameters and reproduction, both in susceptible strains and those resistant to the commercial ixodicides used in México (Pérez et al., 2012; Prado-Ochoa et al., 2013). Both carbamates decrease oviposition and completely inhibit *R. microplus* egg hatching. The eggs produced by treated engorged female ticks had a dark, dry and opaque appearance and were not viable. No studies have described the action mechanism of these carbamates; therefore, the way in which they induce the observed alterations is unknown.

Organophosphates and some carbamates, such as carbaryl and propoxur, act to reversibly inhibit the acetylcholinesterase enzyme (AChE) in the tick nervous system (Tan et al., 2011; Sogorb and Vilanova, 2002). In earlier studies, the compounds LQM 919 and LQM 996 did not have paralyzing effects on larvae (Prado-Ochoa et al., 2013); however, these compounds belong to the carbamate group and thus should be evaluated for their effects on AChE activity in *R. microplus*.

On the other hand, previous studies have demonstrated that carbamates can alter the tick reproduction process; therefore, carbamates likely affect the integrity of the reproductive organs (Prado-Ochoa et al., 2013). Consequently, the aim of the present study was to study the effects produced by the carbamates LQM 919 and LQM 996 on the AChE activity, egg structure and reproductive organs of *R. microplus*.

## MATERIAL AND METHODS

### Carbamates

The carbamates used in this study were designed and synthesized at the Universidad Nacional Autónoma de México, using a benzimidazole molecule as the structural base. The carbamates were synthesized by reacting aryl- and alkylamines with sodium hydride and benzene diethylcarbonate, followed by column chromatography purification; next, the products were recrystallized. The carbamates were structurally characterized through interpretations of their spectra, hydrogen and carbon-13 nuclear magnetic resonance and mass spectrometry (Angeles et al., 2000). The chemical structures, nomenclature, molecular weights and identification codes of the carbamates are shown in Table 1.

### *R. microplus* tick strains

Two *R. microplus* strains were used. The "Media Joya" strain is susceptible to organophosphates, pyrethroids and amidines and is currently used as a reference susceptible strain in Latin America (Aguilar-Tipacamu et al., 2008; Martínez et al., 2008). The "San Alfonso" strain is resistant to organophosphates, pyrethroids and amidines (Soberanes-Céspedes et al., 2002; Alonso-Díaz et al., 2006; Domínguez-García et al., 2010;). The *R. microplus* strains were maintained in Aberdeen Angus breed bovines. The engorged female ticks were collected after spontaneous detachment and incubated for 15 days at 28°C with 80% relative humidity; the produced egg masses were incubated under the same conditions until larvae were obtained. This study was approved by the Internal Committee for Care of Experimental Animals of the Postgraduate Program of Animal Production and Health (UNAM, México). The strains were donated by the National Animal Health Verification Services Center of the State Animal Health Office (SAGARPA) of México.

**Experimental design for the evaluation of AChE activity**

*R. microplus* larvae were obtained as described by Prado-Ochoa et al. (2013). The effects of different concentrations of the studied carbamates, as well as propoxur (2-2(1-methylethoxyl) phenyl methyl carbamate, 99% pure) as a positive control, on mortality were evaluated on the "Media Joya" strain larvae by the Larval Packet Test (LPT) technique. Other larvae were processed for AChE extraction and to evaluate the activity and inhibition kinetics produced by LQM 919, LQM 996 and propoxur. The concentrations that affected the larvae were compared to those that reduced AChE activity.

**Experimental design for evaluations of the effect on morphology**

Engorged female ticks from the susceptible and resistant strains, which spontaneously detached from the host, were treated with both carbamates in an Adult Immersion Test (AIT). On days 1, 3, 5 and 7 post-treatment (p.t.), 30 ticks from each strain (10 treated with LQM 919, 10 treated with LQM 996 and 10 treated with dimethylsulfoxide [DMSO] as an untreated control) were dissected and the reproductive organs were observed. Afterwards, the ticks were processed for conventional histology.

Additionally, 90 engorged female ticks from each strain were divided into three groups as follows (n=30): the first group was treated (AIT) with LQM 919, the second was treated with LQM 996 and the last was treated with DMSO (carbamates-untreated control). On day 7 post treatment (p.t.), the egg masses produced by the females from each group were collected. The collected eggs were measured and processed for scanning electron microscopy.

**Bioassays**

The LPT was performed as described by Stone and Haydock (1962). Groups of larvae from the "Media Joya" strain (susceptible) were treated in triplicate with

different concentrations of LQM 919, LQM 996 and propoxur (positive control). Diluent (trichloroethylene and olive oil, 2:1) without carbamates was used as a negative control. The concentrations (w/v) of each carbamate were 2, 1, 0.5, 0.25 and 0.125%. The concentrations of propoxur were 0.125, 0.0625, 0.0312, 0.015, 0.0078, 0.0039, 0.0019 and 0.0009%. The obtained larvae mortality data were analyzed according to the probit method to estimate the lethal concentrations to 99% of the ticks ($LC_{99}$).

The AIT was performed according to the technique that was described by Drummond (1976) and modified by Prado-Ochoa et al (2013). Carbamates were tested in triplicate at a concentration of 1 mg/ml in all assays.

**Enzyme extraction**

AChE was extracted from 0.1 g of susceptible strain *R. microplus* larvae according to the Li et al. (2005) technique. Larval maceration was performed in tick extraction buffer (10 mM $NaPO_4$, pH 6.5, with 20% sucrose, 1 mM EDTA and 0.5% Triton X-100), and sonication was later performed with 2 pulses for 10 seconds and 50% amplitude (Pruett and Pound, 2006). The protein concentrations of the larval extracts were measured according to the Bradford method (1976). The final protein concentrations were adjusted to 1 µg/µL.

**Determination of AChE kinetics**

AChE activity in *R. microplus* larval extracts was determined according to the spectrophotometer method published by Ellman et al. (1961) and modified by Li et al. (2005). The rate of reaction was monitored at 405 nm and 30°C, and seven absorbance readings were collected with an Ascent ELISA plate reader (Labsystems®) at two-minute intervals. The kinetic constants $K_m$ (Michaelis-Menten constant) and $V_{max}$ (maximum velocity) were obtained by non-linear regression with the least-square algorithms to fit the Michaelis-Menten function. AChE activity was converted to moles of hydrolyzed acetylthiocholine per minute

by dividing the value $V_m$(ΔOD/min) by the molar extinction coefficient $\epsilon_{412} = 1.36 \times 10^4$ $M^{-1}$ $cm^{-1}$ (Chen et al., 2001). All determinations were performed in quadruplicate.

**Inhibition of AChE activity**

The AChE inhibition kinetics in *R. microplus* due to carbamates was determined according to the Chen et al. (2001) method. The AChE inhibition rate was measured in quadruplicate in the presence of $1.2 \times 10^{-4}$ M of acetylthiocholine, 20 µL of larval extract and carbamate concentrations of 50, 25, 12.5, 6.25, 3.13 and 1.56 mM. Larval extract without an inhibitor was used as a negative control. The AChE inhibition kinetics produced by the carbamates were compared to those of propoxur at concentrations of 0.25, 0.05, 0.025, 0.005, 0.0025 and 0.0005 mM. The inhibition kinetics were monitored at 405 nm and 30°C, and seven absorbance readings were collected at two-minute intervals (Pruett, 2002). The biomolecular reaction constant ($k_i$), dissociation constant ($K_d$) and carbamylation constant ($K_2$) were calculated as described by Chen et al. (2001) and Pruett (2002).

To calculate the rate of activity reduction, the inhibition assay was increased to 16 readings at two-minute intervals, and the following equation was used:

$$\% \text{ AChE activity reduction} = 100 - (V_{[I]} / V \text{ x } 100)$$

$V$= AChE activity without inhibitor. Slope of the reaction rate is created by an increase in absorbance over time.

$V_{[I]}$ = AChE activity for each inhibitor concentration. Slope of the reaction rate is created by an increase in absorbance over time.

**Egg measurement**

The eggs obtained from the LQM 919, LQM 996 and DMSO-treated "Media Joya" and "San Alfonso" strain ticks were measured with Image Pro-Premier software (Media Cibernetics®). One hundred and fifty eggs from the carbamate-treated females and 300 eggs from the control females were measured.

**Scanning electron microscopy (SEM)**

The eggs were fixed in 2.5% glutaraldehyde for 2 h, post-fixed in 1% osmium tetroxide for 2 hours and dehydrated in a graded ethanol series (10-100%). The material was processed via critical point drying and the samples were mounted in an aluminum sample holder with a double-sided carbon adhesive and ionized with gold. The eggs were examined and photographed under a Jeol JSM 5410LV scanning electron microscope.

The eggs were fixed in 2.5% glutaraldehyde for 2 h, post-fixed in 1% osmium tetroxide for 2 hours and dehydrated in a graded ethanol series (10-100%). The material was processed via critical point drying and the samples were mounted in an aluminum sample holder with a double-sided carbon adhesive and ionized with gold. The eggs were examined and photographed under a Jeol JSM 5410LV scanning electron microscope.

**Macroscopic and histological observation**

The engorged female ticks were refrigerated for 20 minutes at 4°C to induce thermal shock anesthesia; later, the dorsal cuticles were removed and the internal organs were washed with physiological saline solution to remove the gut contents and hemolymph. Morphological alterations to the reproductive organs were described. The ticks were fixed in 4% paraformaldehyde, processed according to conventional histological techniques and stained with hematoxylin and eosin.

Comparative ovarian morphological studies were performed according to the criteria and terminology proposed by Saito et al. (2005).

**Statistical analysis**

The inhibition kinetic constants of AChE ($k_2$, $k_d$, $k_i$), the percent reduction in AChE activity and the egg lengths were analyzed by one-way ANOVA and Tukey's test for comparisons between the means with a 95% confidence limit. Data for the constants $k_2$, $k_d$ and $k_i$ were logarithmically transformed for normalization prior to their analysis. In the LPT, the $LC_{99}$ was estimated with a Probit analysis (dose-response) and a 95% confidence limit, using Polo-Plus software (Le-Ora-Software, 2004).

**RESULTS**

**AChE inhibition**

The $V_{max}$ and $K_m$ values for AChE in *R. microplus* were the following: $1.24 \times 10^{-6}$ ($\pm$ $0.49 \times 10^{-6}$) M/min/L and $4.91 \times 10^{-5}$ ($\pm$ $2.76 \times 10^{-5}$) M, respectively. The kinetic parameters of the effects of carbamates on AChE in *R. microplus* are shown in Table 2. The $k_i$ values of LQM 919 and LQM 996 on AChE were lower ($P < 0.05$) than those of propoxur. The dissociation of the inhibitor-enzyme complex ($k_d$) was higher ($P < 0.05$) with the carbamates LQM 919 and LQM 996 than with propoxur. The $k_2$ values of AChE, which were inhibited by carbamates LQM 919, LQM 996 and propoxur, were not significantly different ($P > 0.05$).

The effects of LQM 919, LQM 996 and propoxur on the reduction of AChE activity in *R. microplus* larvae are shown in Figure 1. The results show that carbamates reduce AChE activity in *R. microplus* ($P < 0.05$). LQM 919 and LQM 996 reduced AChE activity by less than 40%, while propoxur reduced AChE activity by 99%.

**Larval packet test**

LQM 919 and LQM 996 did not cause the paralysis or death of *R. microplus* larvae at the highest concentration tested (2% active ingredient). Propoxur caused up to 100% mortality in *R. microplus* larvae ($LC_{99}$ = 0.008%, Cl = 0.007-0.010).

**Morphological studies of the eggs**

The eggs that were oviposited by the LQM 919 and LQM 996-treated ticks were smaller ($P < 0.05$) than those of the carbamates-untreated control group (Table 3).

A superficial waxy layer that formed irregular drops on the eggs from carbamates-untreated ticks was observed by SEM; this layer was absent or poorly observed in eggs from LQM 919 or LQM 996-treated ticks (Figure 2).

**Morphology of the reproductive organs**

The untreated and treated ticks remained alive until the end of the experimental observation (when they were anesthetized and dissected). Similar morphological alterations were observed in both strains. On days 1 and 3 p.t., there were no differences in the stereoscopic observations between the reproductive organs from LQM 919 or LQM 996-treated engorged females and those from the control group. On day 5 p.t., reduced ovarian development, a majority of oocytes in the initial developmental stages and few apparently mature oviductal oocytes were observed in the treated ticks. In contrast, a larger quantity of mature oocytes in the ovaries and oviducts full of mature oocytes were observed in the untreated ticks (Figure 3). There were no morphological differences in the size or the glandular development of the Gene's organ among carbamate-treated and untreated ticks.

**Histology of the reproductive organs**

The total of carbamate treated ticks showed histological differences at the ovarian embryo development level with regards to the untreated ticks. The severity and distribution of histological alterations observed in response to the carbamates LQM 919 or LQM 996 were similar in the "Media Joya" and "San Alfonso" strains. On day 1 p.t., cytoplasmic vacuoles in the previtellogenic oocytes (l and ll), a loss of germinal vesicle shape and membrane alterations with degenerative signs were observed in the ovaries of carbamate-treated engorged female ticks. Additionally, decreased chorion deposition and altered granularity were observed in the vitellogenic oocytes (lll and lV) of treated engorged female ticks (Figure 4). Cellular vacuolation was observed in the ovarian epithelium. These alterations persisted and increased in severity until day 7 p.t.

On day 3 p.t., in addition to the above-described alterations, severe ovarian epithelial vacuolation, clear germinal vesicle degeneration and nucleolar fragmentation were observed in the treated oocytes (Figure 5).

On day 5 p.t., only stage IV and V oocytes were observed in the carbamate-untreated ticks (Figure 6A). Oocytes from the treated ticks were altered in shape, ranging from round to irregular, and displayed invaginated plasma membranes (Figure 6B); few oocytes were the final maturation stages.

On day 7 p.t., a clear decrease in oocytes in the final stages of maturation, poor chorion deposition and the presence of a large quantity of degenerated oocytes with large amounts of cytoplasmic vacuoles (Figure 6C) were observed in the ovaries of the treated ticks. The ovarian epithelium was flattened and eroded with pyknotic nuclei (Figure 6D).

## DISCUSSION

In early studies, the carbamates LQM 919 and LQM 996 were shown to affect the development of *R. microplus*, and their use in tick control is promising (Pérez et al., 2012; Prado-Ochoa et al., 2013). Other carbamates such as propoxur or carbaryl act to inhibit AChE in the arthropod nervous system, leading to paralysis and death (Sogorb and Vilanova, 2002; Tan et al., 2011). The results of this study show that the effects of the studied carbamates on *R. microplus* are independent of AChE inhibition and suggest that the morphological alterations observed in the reproductive organs are due to carbamate actions on vitellogenesis and ovarian cell viability.

The $k_i$ (bimolecular reaction constant) is considered the most reliable criterion with which to evaluate the inhibitory power of some substances over AChE (Yi et al., 2006). The present study shows that the studied carbamates, although they reduced the *R. microplus* "Media Joya" strain AChE activity by as much as 40%, had lower $k_i$ values ($P < 0.001$) than those of propoxur, a potent AChE inhibitor (approximately 10,000 times less). The aforementioned shows that carbamates LQM 919 and LQM 996 are weak inhibitors of AChE.

The affinity of a compound for AChE can be measured by the rate at which the compound inhibits its activity, the rate of inhibitor-AChE complex formation and enzyme carbamylation (Pruett and Pound, 2006). The $k_d$ values (dissociation constant) are directly proportional to the carbamate concentration required to inhibit AChE; therefore, an increase in $k_d$ reflects a reduced affinity of carbamate for AChE (Yi et al., 2006). The $k_2$ (carbamylation constant) reflects the rate of inhibitor-AChE complex formation, for which changes in the $k_2$ reflect decreases or increases in the carbamylation velocity. In this study, although the $k_2$ values of the carbamates LQM 919 and LQM 996 were similar to that of propoxur, the $k_d$ calculated in the carbamate assays was greater ($P < 0.001$) than that in the propoxur assays. The

aforementioned finding is indicative of the low affinity that these carbamates hold for *R. microplus* AChE.

A 90% inhibition of AChE activity was shown to be compatible with life when mites were exposed to different compounds (Smissaert et al., 1975). LQM 919 and LQM 996 did not induce larval paralysis or death in the LPT bioassay, even at the highest concentrations (2%; 82 mM and 100 mM, respectively). In the *in vitro* assay, the highest concentrations of these carbamates (50 mM) produced a less than 40% reduction in AChE activity. The latter results show that the weak effects of these carbamates on larval AChE are insufficient to cause death.

Using AIT, the present study has demonstrated that LQM 919 and LQM 996 affect the oviposition and hatching of *R. microplus*. The concentrations at which hatching was inhibited by 99% ($CIE_{99}$) were 2.81 mM for LQM 919 and 1.39 mM for LQM 996 (Prado-Ochoa et al., 2013). In the *in vitro* assay to determine the effects of these carbamates on AChE, concentrations between 1.56 and 12.5 mM did not reduce AChE activity in the larval extracts. The aforementioned finding shows that the mechanism by which these carbamates affect *R. microplus* reproductive parameters is independent of AChE inhibition.

*R. microplus* females oviposit approximately 3000 eggs during their life cycle, thus ensuring larval establishment on the pastures to continue the biological cycle. A critical part of the biotic potential of these ticks is also based on egg viability. Early results obtained by the authors have been confirmed in this study (Prado-Ochoa et al., 2013). Specifically, eggs produced by LQM 919 and LQM 996-treated engorged females from both strains had a dark, dry and opaque appearance, relative to those produced by untreated females and compromised viability. It was also observed that egg adherence decreased and the eggs were not viable. Additionally, the oviposited eggs from both strains of carbamate-treated ticks were smaller ($P < 0.05$) than eggs from untreated ticks, suggesting developmental

alterations. The absence of the waxy layer (seen by SEM) on eggs from treated ticks was not associated with alterations to Gene's organ, as reported by Booth et al., (1986) with precocene, but the absence of this layer explains the low adhesion observed between the eggs and the possible reduction in viability.

In tick eggs, the chorion is the outermost membrane and begins to form in stage III oocytes. One function of this layer is protection against mechanic stimuli, drying and predation while allowing gas exchange for embryonic oxygenation (Hinton, 1982). In carbamate-treated females, a significant decrease in oocyte chorion deposition was histologically observed; this could explain the morphologic changes observed in the eggs, since this might alter eggshell permeability and cause greater egg dryness.

Ovarian development was reported to peak in ixodid ticks in response to nervous system stimulation, which in turn promotes an increase in ecdysteroids, mainly 20-hydroxyecdysone (vitellogenesis hormone), in the hemolymph that ends when the females are fully engorged (replete) and detach from the host. After this event, the nervous system likely does not influence the reproductive system (Friesen and Kaufman, 2009; Weiss and Kaufman, 2001). The present study evaluated the effects of carbamates on engorged female ticks that had spontaneously detached from the host, in which the observed effects on the reproductive organs were likely not associated with nervous system effects and were instead due to the direct action of carbamates on some critical ovarian cells, as confirmed by our histological observations.

The *R. microplus* ovary consists of a horseshoe-shaped, single tubular structure that comprises a lumen delimited by an epithelial cell wall and many oocytes of different developmental stages that join to the ovary through pedicel cells. Oocytes have been classified from I to V according to the developmental stage (Saito et al., 2005). In this study, it was stereoscopically and histologically observed

that the ovaries from carbamate-treated ticks had relatively low quantities of stage IV-V oocytes and high quantities of stage I-II oocytes. Also, changes in the shape and size of yolk granule formation were observed in the oocyte cytoplasm; such changes have been associated with a lack of vitelline development (Oliveira et al., 2009; Roma et al., 2010; Denardi et al., 2011). In ticks, low quantities of oocytes in advanced developmental stages and alterations in granularity indicated the effects of carbamates on vitellogenesis and oocyte maturation.

The oocytes are attached to the ovarian wall through prolongations of the epithelium called pedicels, the main functions of which are the support and production of elements that are later incorporated to the oocyte (Saito et al., 2005). It was observed that carbamates induce severe ovarian epithelial cell vacuolization during the first p.t. days and cell death with nuclear pyknosis at day 7 p.t; this indicates that other functions such as pedicel formation and, subsequently, oocyte nutrition might be compromised in carbamate-treated ticks and thus might contribute to the observed damage.

Oliveira et al. (2009) and Arnosti et al. (2011) have associated the presence of oocyte cytoplasmic vacuoles in fipronil and ricinoleic acid ester-treated ticks with autophagic processes and suggest that these processes are involved in mechanisms to eliminate toxic products (detoxification) or large amounts of damaged organelles. The oocytes from carbamate-treated ticks had large quantities of cytoplasmic vacuoles with basophilic contents, which were associated with degenerative changes and could be an attempt by the cells to counteract carbamate-induced damage.

The decreased oocyte size, nucleolar fragmentation and membrane alterations suggest characteristics of apoptotic processes, although other processes of cell death such as necrosis or autophagy could be present in the elimination of damaged ovarian cells. It is necessary to know whether the studied carbamates can induce apoptosis in tick ovarian cells, as well as associated cellular mechanisms.

The main target of action for some ixodicides, including permethrin, ivermectin and fipronil, is the tick nervous system; however, there are reports of negative effects on the oocytes of ticks treated with sublethal concentrations of these ixodicides (Hamdy et al., 2003; Oliveira et al., 2009; Roma et al., 2010; Roma et al., 2011). The results of this study show that the main effects of the evaluated carbamates are oocyte damage, oogenesis inhibition and the inhibition of oocyte maturation. These effects are responsible for reductions in the quantity and viability of the eggs produced by treated females, as well as the observed morphologic changes.

The relative ease of synthesis of these carbamates (Angeles et al., 2000), the high *in vitro* efficacy against both, susceptible and resistant to conventional ixodicides *R. microplus* strains (Pérez et al., 2012; Prado-Ochoa et al., 2013), the low toxicity in mammals (Prado-Ochoa et al., 2012) and the special target-of-action observed in this study, show the potential use of the evaluated carbamates in tick control.

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## TABLES AND FIGURES

Table 1. Chemical structures and molecular weights of the evaluated carbamates.

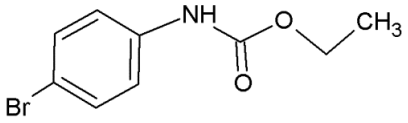
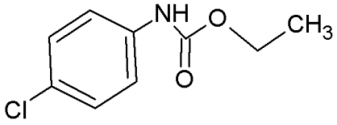
| Carbamate identification code | Chemical structure | Molecular weight (g/mol) |
|---|---|---|
| LQM 919 |  Ethyl (4-bromophenyl)carbamate | 244 |
| LQM 996 |  Ethyl (4-chlorophenyl)carbamate | 199.63 |

Table 2. Kinetic constants for the inhibition (mean±SD) of *Rhipicephalus microplus* AChE by different carbamates ($k_2$= carbamylation constant, $k_d$= dissociation constant and $k_i$= bimolecular reaction constant)

| Carbamate | $k_i$± SD ($M^{-1}$) | $k_d$ ± SD (M) | $k_2$± SD (x $10^{-3}$ $min^{-1}$) |
|---|---|---|---|
| **LQM 919** | 3.62 ± 2.46[a] | 2.90 ± 3.26 (x $10^{-3}$)[a] | 8.98 ± 9.67[a] |
| **LQM 996** | 1.47 ± 0.66[a] | 4.27± 3.33 (x $10^{-3}$)[a] | 13.83± 12.07[a] |
| **Propoxur** | 4.45± 1.19 (x $10^{+4}$ )[b] | 7.64 ± 1.92 (x $10^{-7}$)[b] | 29.48 ± 2.84[a] |

Different letters indicate significant differences between the means ($p<0.05$).

Table 3. Means (±SD) of the lengths (µm) of the eggs oviposited by *Rhipicephalus microplus* engorged females that had been treated with different carbamates.

| | **Susceptible strain** | **San Alfonso strain** |
|---|---|---|
| **Control** | 528.7 ± 28.18[a] | 503.2 ± 16.79[b] |
| **LQM 919** | 438.6 ± 34.95[c] | 429.6 ± 25.52[c] |
| **LQM 996** | 414.1± 33.55[c] | 418.9 ± 24.36[c] |

Different letters indicate significant differences between the means ($p<0.05$).

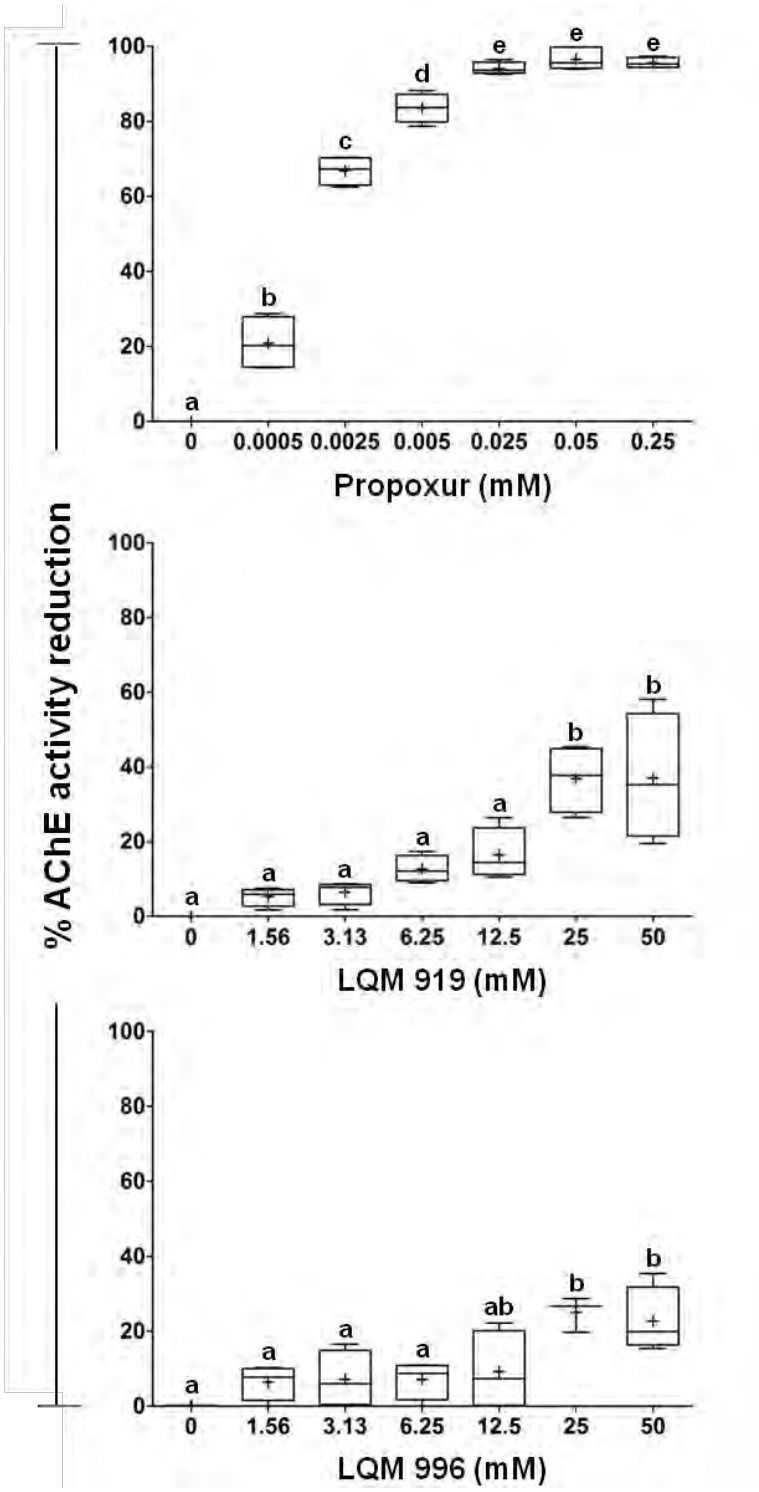



Figure 1. Effects of different carbamates on AChE activity reduction (as a percentage of the control group activity) in the *Rhipicephalus microplus* larval extracts. Different letters indicate significant differences between the means ($p<0.05$).

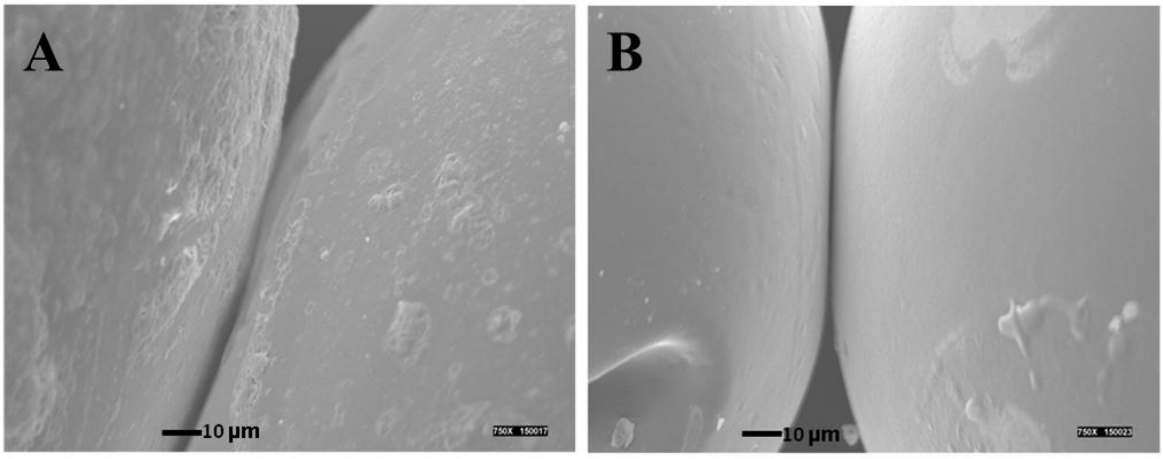



Figure 2. Scanning electron microscopy of eggs produced by *Rhipicephalus microplus* females. A, eggs produced by control female ticks. B, eggs produced by female ticks treated (Adult Immersion Test) with 1 mg/ml of LQM 919.

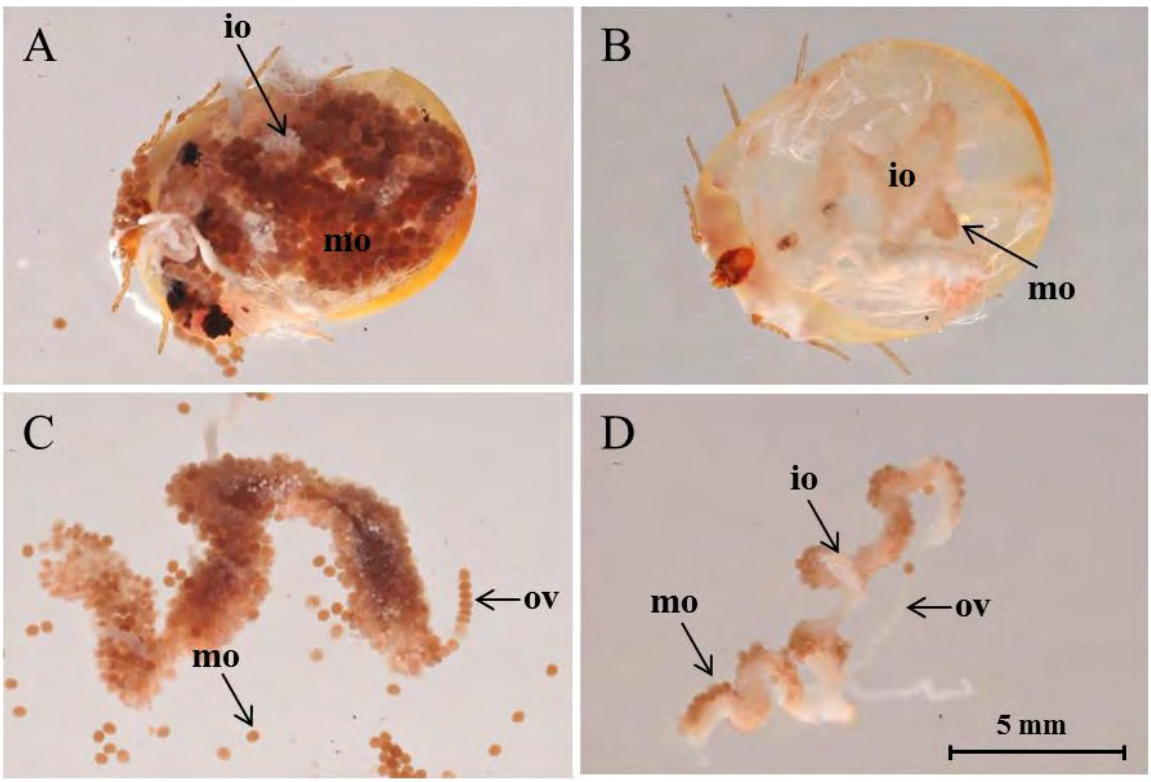



Figure 3. Stereoscopic observation of the reproductive organs from *R. microplus* engorged female ticks at 5 days post-treatment by Adult Immersion Test. A, Dorsal view of an untreated engorged female tick (control). B, Dorsal view of an engorged female tick treated with LQM 996. C, Ovary from an untreated engorged female tick (control). D, ovary from an engorged female tick treated with LQM 996. mo = mature oocytes; io = immature oocytes; ov = oviduct.

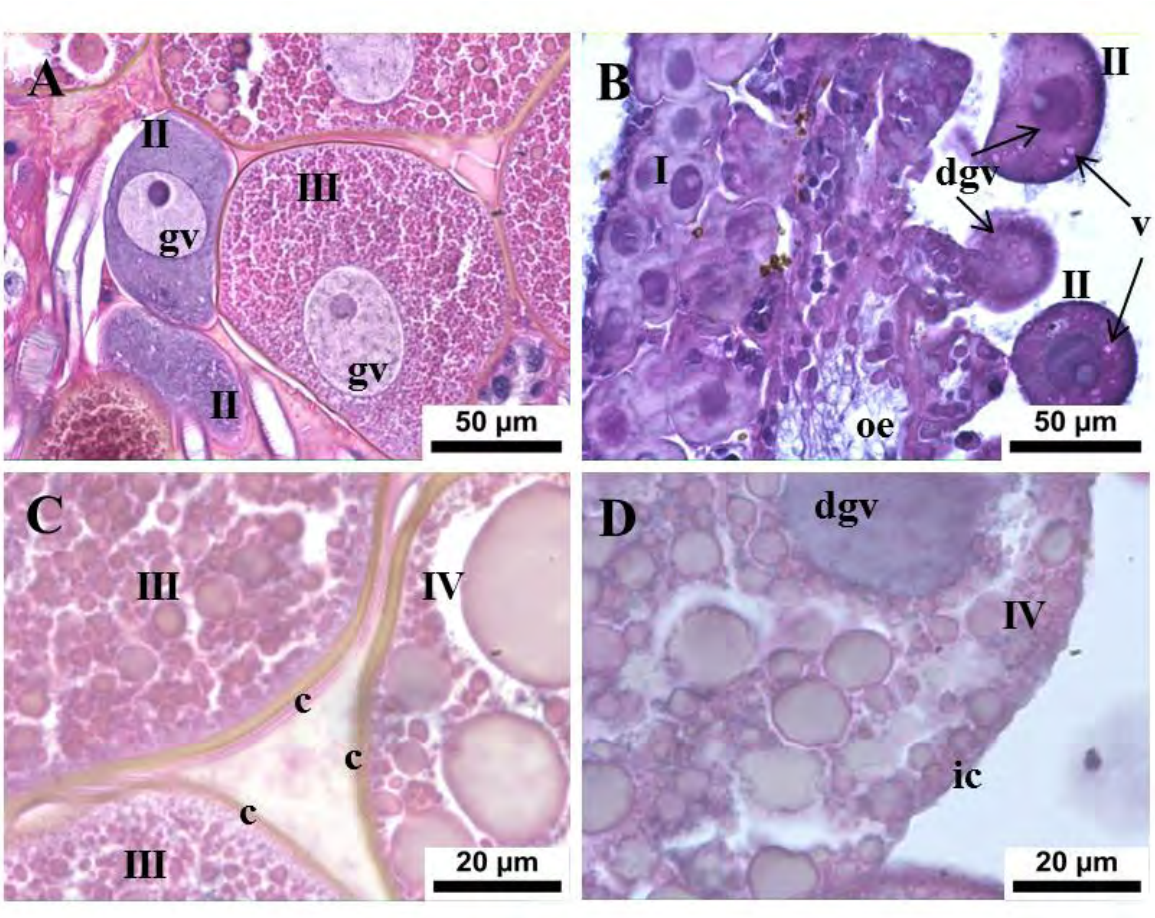



Figure 4. Histological sections (HE-stained) of ovaries from *Rhipicephalus microplus* engorged females at 1 day post-treatment by AIT. A and C, oocytes from a control female tick. B and D, oocytes from a female tick treated with 1 mg/ml of LQM 919. I = oocyte I; II = oocyte II; III = oocyte III; IV = oocyte IV; gv = germinal vesicle; dgv = degenerated germinal vesicle; v = vacuole; oe = ovarian epithelium; c = chorion; ic = incompletely deposited chorion.

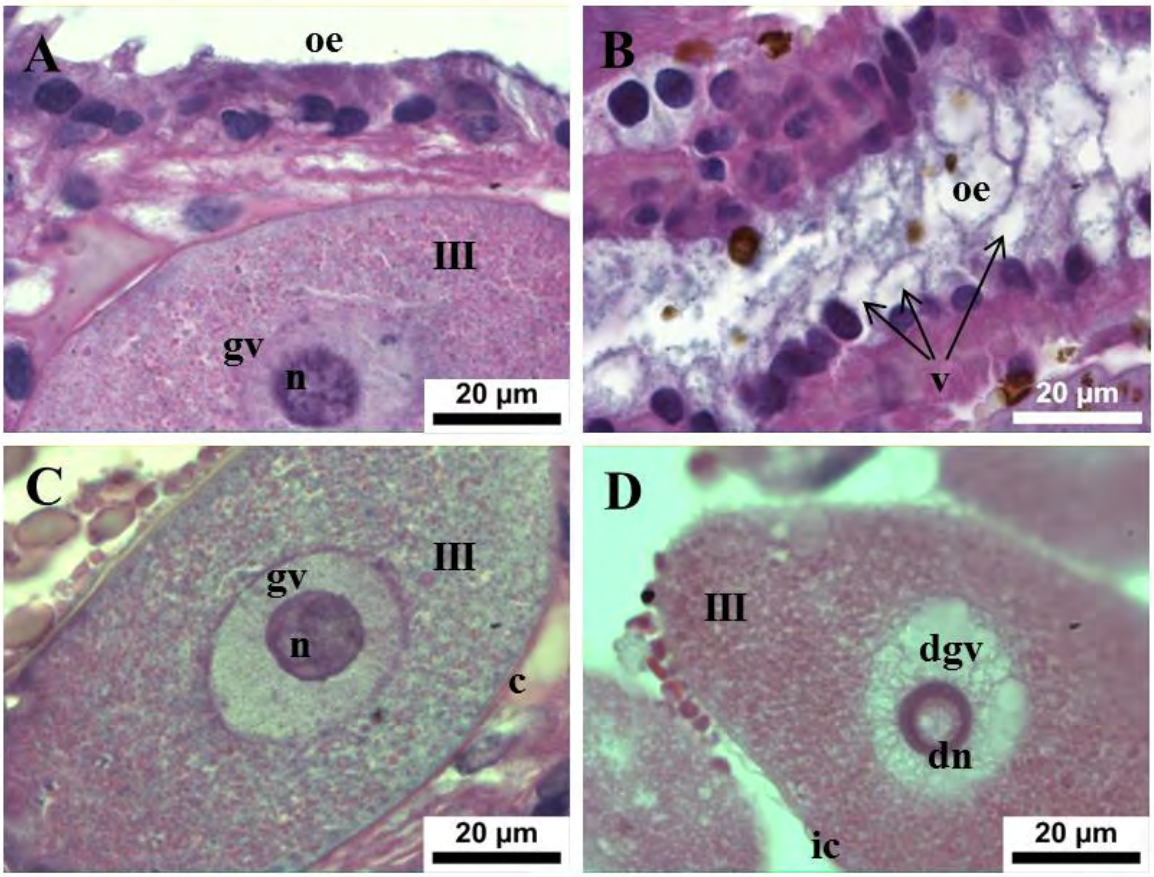



Figure 5. Histological sections (HE-stained) of ovaries from *Rhipicephalus microplus* engorged females at 3 day post-treatment by Adult Immersion Test. A and C, oocytes from a control female tick. B and D, oocytes from a female tick treated with 1 mg/ml of LQM 919. III = oocyte III; gv = germinal vesicle; dgv = degenerated germinal vesicle v = vacuoles; oe = ovarian epithelium; c = chorion; ic = incompletely deposited chorion; n = nucleoli; dn = degenerated nucleoli.

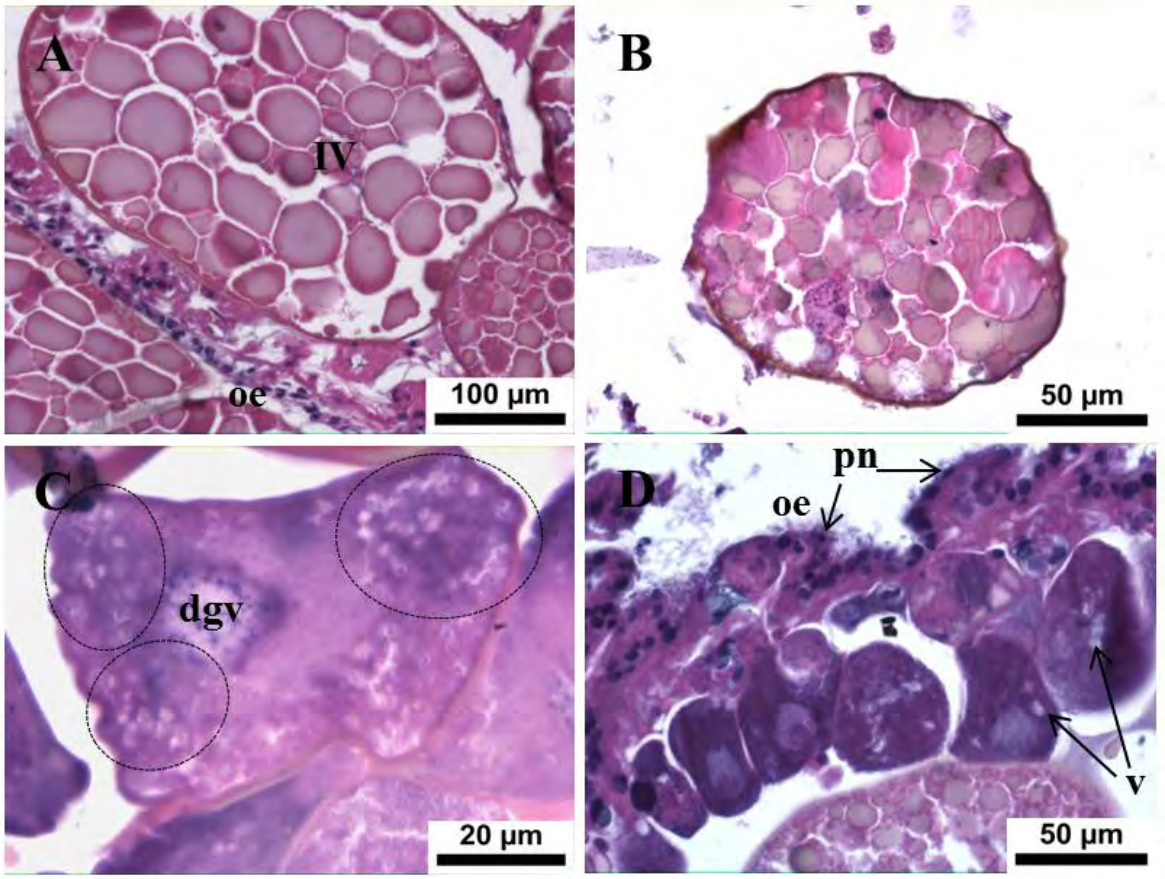



Figure 6. Histological sections (HE-stained) of ovaries from *Rhipicephalus microplus* engorged females at 5 (A and B) and 7 (C and D) days post-treatment by Adult Immersion Test. A, oocytes from a control female tick. B, degenerated oocyte exhibiting membrane invaginations from a female tick treated with 1 mg/ml of LQM 919. C, degenerated oocyte exhibiting prominent vacuolation from a female tick treated with 1 mg/ml of LQM 919. D, degenerated oocytes from a female tick treated with 1 mg/ml of LQM 919. IV = oocyte IV; dgv = degenerated germinal vesicle; v = vacuoles; oe = ovarian epithelium; pn = pyknotic nuclei. Dashed circles indicate the vacuolated areas.

## REFERENCES

Aguilar-Tipacamu, G., Miller, R.J., Hernandez-Ortíz, R., Rodríguez-Vivas, R.I., Vásquez-Pelaez, C., García-Vázquez, Z., Olvera-Valencia, F., Rosario-Cruz, R., 2008. Inheritance of pyrethroid resistance and a sodium channel gene mutation in the cattle tick *Boophilus microplus*. Parasitol. Res.103, 633-639.

Alonso-Díaz, M., Rodríguez-Vivas, R., Fragoso-Sánchez, H., 2006. Resistencia de la garrapata *Boophilus microplus* a los ixodicidas. Arch. med. Vet. 38, 105-113.

Angeles, E., Mart nez, P., Keller, J., Martínez, R., Rubio, M., Ramírez, G., Castillo, R., López-Castañares, R., Jiménez, E., 2000. Ethyl and methylphenylcarbamates as antihelmintic agents: theoretical study for predicting their biological activity by PM3. J. Mol. Struct.: THEOCHEM 504, 141-170.

Arnosti, A., Brienza, P.D., Furquim, K.C., Chierice, G.O., Bechara, G.H., Calligaris, I.B., Camargo-Mathias, M.I., 2011.Effects of ricinoleic acid esters from castor oil of *Ricinus communis* on the vitellogenesis of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) ticks. Exp. Parasitol.127, 575-580.

Booth, T.F., Beadle, D.J., Hart, R.J., 1986. The effects of precocene treatment on egg wax production in Gene's organ and egg viability in the cattle tick *Boophilus microplus* (Acarina Ixodidae): an ultrastructural study. Exp. Appl. Acarol. 2, 187-198.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Chen, Z., Newcomb, R., Forbes, E., McKenzie, J., Batterham, P., 2001.The acetylcholinesterase gene and organophosphorus resistance in the Australian sheep blowfly, *Lucilia cuprina*. Insect. Biochem. Mol. Biol. 31, 805-816.

Denardi, S.E., Bechara, G.H., de Oliveira, P.R., Camargo Mathias, M.I., 2011. Inhibitory action of neem aqueous extract (*Azadirachta indica* A. Juss) on the vitellogenesis of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) ticks. Microsc. Res. Tech. 74, 889-899.

Domínguez-García, D.I., Rosario-Cruz, R., Almazán-García, C., Oaxaca, J.A.S., De la Fuente, J., 2010.Resistencia a los acaricidas y su impacto en la salud animal. Trop. Subtrop. Agroecosys.12, 181-192.

Drummond, R.O., Ernst, S.E., Trevino, J.L., Gladney, W.J., Graham, O.H., 1976. Tests of acaricides for control of *Boophilus annulatus* and *B. microplus*. J. Econ. Entomol. 69, 37-40.

Ellman, G.L., Courtney, K.D., Andres, V., Jr., Feather-Stone, R.M., 1961.A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-95.

Friesen, K.J., Kaufman, W.R., 2009. Salivary gland degeneration and vitellogenesis in the ixodid tick *Amblyomma hebraeum*: Surpassing a critical weight is the prerequisite and detachment from the host is the trigger. J. Insect. Physiol. 55, 936-942.

Hamdy, H.S., Aleya, S.M., Ashraf, A., 2003. Ultrastructural and histological changes induced by ivermectin in the ovary of *Argas persicus* after feeding. Egypt. J. Hosp. Med.10, 154-172.

Hinton, H.E. 1982. Biology of Insect Egg Shells. Pergamon, Oxford.

Le-Ora-Software, 2004. A user´s guide to probit or logit analysis. Le Ora Software. CA, USA: Berkeley.

Li, A.Y., Pruett, J.H., Davey, R.B., George, J.E., 2005. Toxicological and biochemical characterization of coumaphos resistance in the San Roman strain of *Boophilus microplus* (Acari: Ixodidae). Pest. Biochem. Physiol. 81, 145-153.

Martínez, I.F., Ortiz, N.A., Osorio, M.J., 2008. Comportamiento toxicológico a organofosforados y piretroides de *Boophilus microplus* cepa susceptible (CENAPA) del año 2002 al 2005, posterior a su caracterización. In: Memorias del VI Seminario Internacional de Parasitología Animal, Boca del Río, Ver, México.

Oliveira, P.R., Bechara, G.H., Morales, M.A., Mathias, M.I., 2009. Action of the chemical agent fipronil on the reproductive process of semi-engorged females of the tick *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae).

Ultrastructural evaluation of ovary cells. Food. Chem.Toxicol. 47, 1255-1264.

Pérez, I.E., Prado-Ochoa, M.G., Cuenca-Verde, C., Lara, M., Abrego, H., Velázquez, A.M., Angeles-Anguiano, E., Muñoz-Guzmán, M.A., Alba-Hurtado, F., 2012. Assessment of the effect of new ethyl and methyl carbamates on *Rhipicephalus microplus* resistant to conventional acaricides. In: Memories of XI European Multicolloquium of Parasitology (EMOP), Cluj-Napoca, Romania.

Prado-Ochoa, M.G., Muñoz-Guzmán, M.A., Abrego-Reyes, V.H., Velázquez-Sánchez, A.M., Gutiérrez-Amezquita, R.A., Ramírez-Noguera, P., Angeles, E., Alba-Hurtado, F., 2012. Evaluación de la toxicidad dérmica aguda de 2 nuevos carbamatos con actividad sobre *Rhipicephalus microplus*. In: Memorias del VII Seminario Internacional y IX Congreso Nacional de Parasitología Veterinaria, Querétaro, Qro., México.

Prado-Ochoa, M.G., Muñoz-Guzmán, M.A., Abrego-Reyes, V.H., Velázquez-Sánchez, A.M., Lara-Rocha, M., Cuenca-Verde, C., Angeles, E., Alba-Hurtado, F., 2013. Effect of new ethyl and methyl carbamates on biological parameters and reproduction of the cattle tick *Rhipicephalus microplus*. Vet. Parasitol. 194, 49-57.

Pruett, J.H., 2002. Comparative inhibition kinetics for acetylcholinesterases extracted from organophosphate resistant and susceptible strains of *Boophilus microplus* (Acari: Ixodidae). J. Econ. Entomol. 95, 1239-1244.

Pruett, J.H., Pound, J.M., 2006. Biochemical diagnosis of organophosphate-insensitivity with neural acetylcholinesterase extracted by sonication from the adult tick synganglion. Vet. Parasitol. 135, 355-363.

Roma, G.C., Furquim, K.C., Bechara, G.H., Camargo-Mathias, M.I., 2010. Permethrin-induced morphological changes in oocytes of *Rhipicephalus sanguineus* (Acari: Ixodidae) semi-engorged females. Food. Chem. Toxicol. 48, 825-830.

Roma, G.C., Furquim, K.C., Bechara, G.H., Camargo Mathias, M.I., 2011. Cytotoxic effects of permethrin in oocytes of *Rhipicephalus sanguineus* (Acari: Ixodidae) fully engorged females: I. Direct or indirect action of the acaricide in germ cells? Exp. Appl. Acarol. 53, 287-299.

Saito, K.C., Bechara, G.H., Nunes, E.T., de Oliveira, P.R., Denardi, S.E., Mathias, M.I., 2005. Morphological, histological, and ultrastructural studies of the ovary of the cattle-tick *Boophilus microplus* (Canestrini, 1887) (Acari: Ixodidae). Vet. Parasitol. 129, 299-311.

Smissaert, H.R., el Hamid, F.M., Overmeer, W.P., 1975. The minimum acetylcholinesterase (AChE) fraction compatible with life derived by aid of a simple model explaining the degree of dominance of resistance to inhibitors in AChE "mutants". Biochem. Pharmacol. 24, 1043-1047.

Soberanes-Céspedes, N., Santamaría-Vargas, M., Sánchez-Fragoso, H., García-Vázquez, Z., 2002. Primer caso de resistencia al amitraz en la garrapata del ganado *Boophilus microplus* en México. Téc. Pecu. Méx.40, 81-92.

Sogorb, M.A., Vilanova, E., 2002. Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. Toxicol. Lett. 128, 215-228.

Stone, B., Haydock, K., 1962. A method for measuring the acaricide-susceptibility of the cattle tick *Boophilus microplus* (Can.). Bull. Of Entomol. Res. 53, 563-578.

Tan, F., Wang, L., Wang, J., Wu, X., Zhu, H., Jiang, L., Tao, S., Zhao, K., Yang, Y., Tang, X., 2011. Enhanced pesticide sensitivity of novel housefly acetylcholinesterases: a new tool for the detection of residual pesticide contamination. Bioprocess. Biosyst. Eng. 34, 305-314.

Weiss, B.L., Kaufman, W.R., 2001. The relationship between 'critical weight' and 20-hydroxyecdysone in the female ixodid tick, *Amblyomma hebraeum*. J. Insect. Physiol. 47, 1261-1267.

Yi, M.Q., Liu, H.X., Shi, X.Y., Liang, P., Gao, X.W., 2006. Inhibitory effects of four carbamate insecticides on acetylcholinesterase of male and female *Carassius auratus* in vitro. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 143, 113-116.

# Capítulo 8

## ASSESSMENT OF ACUTE ORAL AND DERMAL TOXICITY OF 2 ETHYL-CARBAMATES WITH ACTIVITY AGAINST *Rhipicephalus microplus* IN RATS

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**SUMMARY**

The acute oral and dermal toxicity of two new ethyl-carbamates (ethyl-4-bromphenyl-carbamate and ethyl-4-chlorphenyl-carbamate) with ixodicide activity was determined in rats. The oral $LD_{50}$ of each carbamate was 300 to 2000 mg/kg, and the dermal $LD_{50}$ of each carbamate was >5000 mg/kg. Clinically, the surviving rats that had received oral doses of each carbamate showed decreased weight gain ($p<0.05$) and had nervous system manifestations. These clinical signs were evident from the 300 mg/kg dose and were reversible, whereas the 2000 mg/kg dose caused severe damage and either caused their death or was motive for euthanasia. At necropsy, these rats had dilated stomachs and cecums with diffuse congestion, as well as moderate congestion of the liver. Histologically, the main organ affected was the liver, in which degenerative lesions were observed together with binucleated hepatocytes, focal coagulative necrosis and congestion areas; the severity of the lesions increased with dosage. Furthermore, an increase in gamma-glutamyltransferase, lactate dehydrogenase and creatinine was observed in the plasma of some rats. The dermal application of the maximum dose (5000 mg/kg) of each carbamate did not cause clinical manifestations or liver and skin alterations. This finding demonstrates that the carbamates under study have a low oral hazard and low acute dermal toxicity.

Keywords: carbamates; oral acute toxicity; dermal acute toxicity; rats; ticks control

## INTRODUCTION

*Rhipicephalus microplus* is the most important tick in tropical and subtropical areas in Mexico and throughout the world, causing great economic losses in livestock production (Oliveira et al., 2005). For many years, the most used strategy for controlling ticks has been the use of chemical ixodicides. Nevertheless, the high selection pressure caused by their exaggerated use has promoted resistance to the main commercial ixodicides (Soberanes Céspedes et al., 2005). This resistance has compelled the development of new pharmaceutical alternatives for the control of ticks. Among these alternatives is the development of new molecules for which ticks have not developed resistance.

Our group has shown that the new carbamates synthesized in FES-Cuautitlan-UNAM, namely ethyl-4-bromophenyl-carbamate (LQM 919) and ethyl-4-chlorphenyl-carbamate (LQM 996), negatively affect *R. microplus* biological parameters and reproduction, both in susceptible strains and in those resistant to the commercial ixodicides used in México (Prado-Ochoa et al., 2013a; Pérez-Gonzalez, et al., 2013). These carbamates caused alterations in the reproductive organs, vitellogenesis and the viability of the ovarian cells, and these effects were found to be independent of acetylcholinesterase inhibition (Prado-Ochoa et al., 2013b).

Before these new carbamates can be considered for use in the control of ticks it is necessary to assess the adverse effects that they could cause in mammals. Previous studies have shown that the toxicity of known carbamates is variable (Baron et al., 1991) Some carbamates are highly toxic, for example aldicarb (2-methyl-2 [methylthio] propionaldehyde o-[methylcarbamoyl] Oxime) which has an oral 50% lethal dose ($LD_{50}$) of 0.3 to 0.9 mg/kg, carbofuran ( 2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) which has an oral $LD_{50}$ of 8 mg/kg and carbaryl (1-naphthyl methylcarbamate) which has an oral $LD_{50}$ of 12.5 mg/kg (U.S. Environmental Protection Agency, 2003). Other carbamates such as propoxur (2-

isopropoxyphenyl methylcarbamate), which has an oral $LD_{50}$ of 68 to 94 mg/kg and dermal $LD_{50}$ of >2000 mg/kg (U.S. Environmental Protection Agency, 1997), are considered to be of mid-level toxicity. In contrast, benzimidazoles show low toxicity (Pan Pesticide Database). Albendazole (5-[propylthio]-1H benzimidazol-2-yl carbamic acid methyl ester) shows an $LD_{50}$ of 1320 – 2400 mg/kg, whereas mebendazole (methyl 5-benzoyl-1H-benzimidazol-2-yl-carbamate) has an oral $LD_{50}$ of 715 to 1434 mg/kg (Dayan, 2003).

In bovines, the proposed administration pathway for the carbamates LQM 919 and 996 is dermal using aspersion or immersion baths. Nevertheless, the dermal pathway also represents the highest risk for human contact with ixodicide products. Furthermore, due to the grooming behavior in bovines, they could ingest the products used in baths. Taking into consideration the aforementioned, in this study we determined the acute oral and dermal toxicity in rats caused by the administration of the two new ethyl-carbamates with inhibitory activity on the embryonic development of *R. microplus.*

## MATERIALS AND METHODS

### Animals

Clinically healthy 7- to 8-week-old male Wistar rats weighing between 175 and 200 g were used. All animals were kept in groups of 5 individuals. The environmental temperature was maintained at 22±2°C with a relative humidity between 30-70% and a 12:12 light: dark cycle. They were fed with commercial feed and water *ad libitum*. This study was approved by the Internal Committee for the Care of Experimental Animals of the Postgraduate Program of Animal Production and Health (UNAM, Mexico).

**Evaluated carbamates**

The carbamates used in this study were designed and synthesized at the Universidad Nacional Autónoma de México, using a benzimidazole molecule as the structural base. The carbamates were synthesized by reacting aryl- and alkylamines with sodium hydride and benzene diethylcarbonate, followed by column chromatography purification. Next, the products were recrystallized. The carbamates were structurally characterized through interpretations of their spectra, hydrogen and carbon-13 nuclear magnetic resonance and mass spectrometry (Angeles et al., 2000).

Since the carbamates used in this study are insoluble in water, they were first dissolved in 1 mL of dimethylsulfoxide (DMSO) and later diluted to 2 mL using corn oil for oral administration or with water for dermal administration. The chemical structure, nomenclature, molecular weight and identification key of the studied carbamates are shown in Table 1.

**Experimental design for acute oral toxicity**

A total of 50 Wistar rats distributed in 10 groups of 5 rats each were used. Rats in groups 1, 2, 3 and 4 received 5, 50, 300 and 2000 mg/kg, respectively, of LQM 919 dissolved in DMSO and corn oil. Rats in groups 5, 6, 7 and 8 received 5, 50, 300 and 2000 mg/kg, respectively, of LQM 996 dissolved in DMSO and corn oil. Rats in group 9 received DMSO dissolved in corn oil whereas rats in group 10 only received corn oil (control groups). Treatments were administered in a single dose using an intragastric tube (OECD, 2001).

Animals were kept under observation from day 5 before treatment until 14 days post-treatment (p.t.). Rats that survived were humanely euthanized on day 14 p.t. (NOM-062-ZOO-1999).

Necropsies were carried out at the time of death or euthanasia and any observed macroscopic alterations were recorded. Samples were also taken from the

lung, brain, cerebellum, intestine, stomach, liver, kidney, heart and muscle. The collected samples were fixed in 4% paraformaldehyde and processed using conventional techniques for histopathological study. Also, blood was collected at the time of death or euthanasia in order to measure biochemical parameters, and liver samples were also used to quantify Thiobarbituric Acid Reactive Substances (TBARS).

**Experimental design for acute dermal toxicity**

A total of 40 male Wistar rats distributed in 8 groups of 5 rats each were used. Rats in groups 1, 2 and 3 received topically 500, 2000 and 5000 mg/kg, respectively, of LQM 919 dissolved in DMSO and water. Rats in groups 4, 5 and 6 received topically 500, 2000 and 5000 mg/kg, respectively, of LQM 996 dissolved in DMSO and water. Rats in group 7 received topically DMSO and water, whereas rats in group 10 only received topical water (control groups). The backs of the rats were shaved 24 hours before the dermal application of the carbamates. At the time of treatment, the corresponding carbamate dose was applied on the intact skin of the rats and a semi-occlusive patch was placed on the application site for an exposure period of 24 hours. At the end of said period, the patch was removed and the exposure area was washed with water in order to remove any product residues.

Animals were kept under observation from day 5 before treatment up until 14 days p.t. All rats were humanely euthanized on day 14 p.t. (NOM-062-ZOO-1999). Necropsies were carried out at the time of death or euthanasia and observed macroscopic alterations were recorded. Samples from lung, brain, cerebellum, intestine, stomach, liver, kidney, heart and muscle were also taken. The collected samples were fixed in 4% paraformaldehyde and processed using conventional techniques for histopathological study. Also, blood was collected at the time of death or euthanasia in order to measure biochemical parameters, as well as liver samples to quantify TBARS.

**Clinical observations**

Rats from both experiments were clinically checked twice a day prior to treatment, then every hour during the first eight hours p.t. and three times a day during the rest of the experiment. Skin, mucosa, eyes, respiratory rate, nasal secretions, salivation, tremors and convulsions, changes in activity levels, posture, gait, sensory reaction to stimulus and strange behaviors were systematically checked following the table proposed by Morton and Griffiths (1985). Furthermore, the presence of erythema and edema in the skin area exposed to carbamates was assessed in rats treated dermally following the scale proposed by Draize et al. (1946). The weights of all rats were recorded at 0, 7 and 14 days p.t.

**Biochemical tests**

Plasma was obtained from the blood samples and frozen at -80ºC until they were processed. Plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and gamma-glutamyltransferase (GGT) were measured using the methods described by Reitman and Frankel (1957), Wooton (1964) and Szasz (1969), respectively. The concentrations of total protein, albumin and plasma creatinine were determined by the methods established by Westgard and Poquette (1972), Gornal (1949) and Bartels (1972), respectively. The aforementioned determinations were carried out using commercial kits from BioSystems®. In order to calculate globulin concentrations in the plasma, the value of albumin was subtracted from that of total protein. The albumin and globulin (A/G) ratio was calculated by dividing the concentration of albumin by the concentration of globulin. Cholinesterase activity (CHE) was determined using the colorimetric method described by Ellman (1961) using a commercial kit from Wiener Lab®.

**TBARS quantification**

Samples (0.5 $cm^3$ in size) were collected from the liver of all rats, submerged in a buffer solution (PBS pH 7.2, 15mM sodium azide, 1mM PMSF, 0.1%Triton X-100 and 5mM EDTA) and frozen in liquid nitrogen until further processing. Tissues were thawed on ice, and the original buffer solution was discarded. Then, recently prepared buffer solution was added and the tissues were then homogenized mechanically, sonicated (3 pulses of 10 seconds, 50% amplitude) and finally centrifuged (13000 g, 7´ to 4ºC). The supernatant (40 µL) was added to an equal volume of 2.5% perchloric acid and incubated at ambient temperature for 10 minutes. Afterwards, samples were centrifuged at 13000 g for 10 minutes at 4ºC and the supernatant was reacted with 0.067% thiobarbituric acid (TBA) at 90ºC for 30 minutes (Coban and Inanc, 2004). The TBARS content of the samples was quantified using a standard curve generated using malondialdehyde (MDA) at 532nm (Lykkesfeldt, 2001). The concentration of TBARS was expressed in nmol/mg of protein.

**RESULTS**

**$LD_{50}$**

The number of rats who died or were euthanized due to clinical signs of severe toxicity due to the oral or dermal administration of the carbamates in this study is shown in Table 2. The $LD_{50}$ for the oral pathway of each carbamate was found to be between 300 and 2000 mg/kg. Also, the $LD_{50}$ for the dermal pathway of each carbamate was >5000 mg/kg.

**Body weight**

The oral administration of 5 and 50 mg/kg of each carbamate did not have an effect on weight gain in treated rats ($P>0.05$). Surviving rats that had received orally 300 mg/kg of carbamates LQM 919 and LQM 996 (39.7 ± 12.1 g and 23 ± 12.5 g,

respectively) showed decreased weight gain ($P<0.01$) when compared to rats in the control groups (corn oil + DMSO 88.6 ± 17.6 g; corn oil 55.6 ± 6.5 g). None of the dosages applied dermally had an effect on the weight gain ($P>0.05$) of treated rats when compared to the control rats (water 52.2 ± 15 g; water + DMSO 51.8 ± 14.5 g).

**Clinical manifestations**

The oral administration of 5 and 50 mg/kg of each carbamate did not produce clinical manifestations associated with toxicity in treated rats. The clinical manifestations observed in rats treated orally with 300 and 2000 mg/kg of each carbamate are shown in Table 3. None of the dosages of each carbamate administered dermally produced clinical manifestations associated with toxicity in rats.

**Findings at necropsy**

Macroscopically, rats that died or that were euthanized after the oral administration of 2000 mg/kg of carbamate LQM 919 only exhibited a dilated stomach and cecum with diffuse congestion. The single rat that survived from this group until the end of the study had moderate congestion of the liver. Two of the rats that received 300 mg/kg and 4 of those that received 2000 mg/kg of carbamate LQM 996 had moderate diffuse congestion of the liver. The remainder of the rats that received the oral dosage together with all rats that received the dermal dosages and the control rats did not show apparent pathological changes at necropsy.

**Histopathology**

Rats from all experimental groups that received oral dosages of carbamate LQM 919 exhibited albuminous degeneration of the liver with the presence of hepatocytes with highly euchromatic nuclei and slight vacuolar degeneration. Furthermore, rats that received the 300 mg/kg dose showed an increase in

hepatocytes with two nuclei and increased liver congestion. Rats that received the 2000 mg/kg dose showed coagulative necrotic foci as well as congestion in their liver, and had hyaline degeneration and pyknotic nuclei (coagulative necrosis) in areas of their renal cortices (Figure 1).

Rats from all experimental groups that received oral doses of carbamate LQM 996 had necrotic foci and highly euchromatic nuclei in their livers; the severity of the lesions increased with increased dosage.

The remainder of the organs in rats that received oral administration of each carbamate did not show microscopic lesions. The rats that received the dermal administration and the rats from control groups did not show apparent microscopic lesions in the organs that were sampled.

**Biochemical tests**

Rats that received orally 300 mg/kg of carbamate LQM 919 had an increase ($P<0.05$) of GGT (2.45 ± 1.87 U/L) at day 14 p.t. when compared to the control group that received corn oil + DMSO (1.06 ± 0.68 U/L). Rats that received orally 2000 mg/kg of said carbamate showed between 3 to 22 hours p.t. (time of death or euthanasia) a higher concentration ($P<0.001$) of LDH (1170 ± 120.6 U/L) when compared to the control group that received corn oil + DMSO (493.4 ± 114.2 U/L). The remainder of the rats that received dosages of carbamate LQM 919 either orally or dermally did not show significant differences ($P>0.05$) in any of the biochemical parameters evaluated when compared to the control group.

Rats that received orally 300 mg/kg of carbamate LQM 996 had an increase ($P<0.001$) in creatinine concentration (1.13 ± 0.64 U/L) at day 14 p.t. when compared to the control group that received corn oil + DMSO (0.49 ± 0.12 mg/dL). Rats that received dermally 5000 mg/kg of said carbamate showed a greater concentration ($P<0.05$) of LDH (1270 ± 652.2 U/L) when compared to the control group that received corn oil + DMSO (603.0 ± 172.1 U/L). The remainder of the rats

that received dosages of carbamate LQM 996 either orally or dermally did not show differences in any of the biochemical parameters evaluated when compared to the control group.

**TBARS**

Rats in the groups that received oral dosages of 5 or 50 mg/kg of carbamate LQM 919 (12.3 ± 9.7 and 8.4 ± 5.4 nmol/mg, respectively) and those that received the 5 mg/kg dosage of carbamate LQM 996 (10.7 ± 8.9 nmol/mg) showed an increase ($P<0.05$) in the concentration of TBARS in the liver when compared to the control rats that received corn oil + DMSO (1.2 ± 1.0 nmol/mg). Rats in the remaining groups that received dosages of each carbamate did not show significant differences ($P>0.05$) when compared to the control groups. Rats of all groups that received a dermal administration of each carbamate did not show differences ($P>0.05$) with the control groups.

**DISCUSSION**

A desirable characteristic of any drug is that it has therapeutic effects at low dosages and has the least amount of undesirable secondary and toxic effects on individuals. Previous work has shown that the synthetic carbamates that were evaluated in this study inhibit the in vitro reproduction of *R. microplus* at low concentrations (Prado-Ochoa et al., 2013a). The results of this study, following the guidelines of the Organization for Economic Cooperation and Development (OECD, 2001), show that the synthetic carbamates evaluated in this study have low toxicity in rats.

The $LD_{50}$ is used as a general indicator of acute toxicity of a substance. Currently, the majority of studies conducted to evaluate the acute toxicity of new pharmaceuticals are based on the Fixed Dose Procedure recommended by the OECD (2001), so this study used said methodology. The oral pathway $LD_{50}$ of both carbamates was 300 to 2000 mg/kg. Taking into account the criteria set by the

Globally Harmonized System (GHS) of Classification and Labeling of Chemicals (UN, 2011), these carbamates can be classified as category 4 (low hazard). Other pesticides that are considered by the GHS to have high oral toxicity such as coumaphos ($LD_{50}$ 13 mg/kg) or be moderately toxic such as diazinon, chlorpyriphos and malathion ($LD_{50}$ 76 mg/kg, 82 mg/kg and 290 mg/kg, respectively) are currently being used commercially (Kegley et al., 2011). In this context, the estimation of the higher $LD_{50}$ of the carbamates evaluated in this study suggest a relative reduced acute hazard in mammals.

The effects of the carbamates evaluated in this study on engorged female ticks in Adult Immersion Tests suggest that they should be used directly on the bovine's skin (Prado-Ochoa et al., 2013a). The dermal application of up to 5000 mg/kg of the carbamates in this study did not cause death in any of the treated rats (dermal $LD_{50}$>5000 mg/kg). Therefore, according to the GHS criteria they should be classified as category 5 (low acute toxicity). The data show that the carbamates evaluated in this study have less dermal toxicity than has been reported in the Pan Pesticide Database for other commercial pesticides (coumaphos, dermal $LD_{50}$ of 860 mg/kg; diazinon, dermal $LD_{50}$ of 455 mg/kg; chlorpyriphos, dermal $LD_{50}$ of 202 mg/kg and malathion, dermal $LD_{50}$ of 4444 mg/kg).

The safety margin of an ixodicide can be estimated indirectly by calculating the relationship between the oral or dermal $LD_{50}$ and the effective concentration on ticks. The ratio between the oral $LD_{50}$ (<300 mg/kg) or dermal $LD_{50}$ (>5000 mg/kg) levels in rats obtained in this study and the concentration that inhibits tick reproduction that has been previously reported (LQM 919= 0.687 mg/mL and LQM 996= 0.279 mg/mL; Prado-Ochoa et al., 2013a) were 436:1 and 7278:1 for LQM 919 (oral and dermal, respectively), and 1075:1 and 17921:1 for LQM 996 (oral and dermal, respectively). If this same calculation method is used for the data found in the literature (Foil et al., 2004; Kegley et al.,2011), then coumaphos has a ratio of 65:1 and 4300:1 (oral and dermal, respectively) and chlorpyriphos has a ratio of

273:1 and 673:1 (oral and dermal, respectively). These data suggest that the carbamates evaluated in this study have a greater safety range than some organophosphates that are currently being used commercially.

The oral administration of high doses (300-2000 mg/kg) of the studied carbamates caused in rats various levels of immobility, prostration, hypothermia, depression of spontaneous and provoked behavior, and paralysis with extension of hind quarters. These signs were evident from the 300 mg/kg dose and were reversible, whereas those that occurred at the 2000 mg/kg dose were severe and either caused their death or were the reason for euthanasia of the rats. The observed clinical signs coincide with the effects caused by other carbamates that reversibly inhibit acetylcholinesterase in the nervous system and cause the accumulation of acetylcholine in cholinergic synapses. Nevertheless, plasma cholinesterase levels did not decrease in treated rats. Furthermore, in vitro observations made by our group showed that the carbamates used in this study are weak inhibitors with a low affinity for acetylcholinesterase in R. microplus (Prado-Ochoa et al., 2013b). In light of this finding, we associate the clinical signs observed in treated rats with the weak inhibitor effect on acetycholinesterase of the carbamates that were evaluated in this study.

The body weight gain of experimental animals is an indicator of the degree of wellness and health of the subjects. The surviving rats at the oral dosage of 300 and 2000 mg/kg of carbamates evaluated in this study exhibited decreased weight gain ($P<0.05$) when compared to rats in the control group. This finding indicates that high oral dosages of the carbamates have an effect on the general wellbeing of the treated rats. In contrast, their dermal application did not have an effect on the weight gain or general condition of rats.

Some enzymes such as AST, ALT, GGT and LDH are used as bioindicators of liver damage. An elevation in their plasma levels is mainly due to the alterations

in the hepatocyte membrane or changes in its permeability. The carbamates that were studied caused a moderate elevation of GGT in rats that received a 300 mg/kg oral dose. The increase in GGT levels indicates that there was damage to hepatocytes, although the absence of increases of the other transaminases suggests that the damage is minor. The enzyme GGT has an important role in antioxidant homeostasis and is frequently upregulated after acute oxidative stress. There is some evidence of the elevation in systemic GGT activity, which is characterized by the extended generation of reactive oxygen species (Irie et al., 2012; Ravuri et al., 2013). In this work, we observed an increase of TBARS in the livers of rats treated orally with the evaluated carbamates. These results suggest the association of toxic effects in rats exposed orally and oxidative damage estimated by the increase in TBARS. More studies are necessary in order to elucidate the action mechanism of the new carbamates and its likely effect on the modulation of oxidative stress. Histologically, they caused liver damage, and the severity of the observed lesions was dose-dependent. Rats that died during the first 24 hours p.t. did not show histological changes in their livers. In contrast, rats that were euthanized at 14 days p.t. had various levels of degenerative and necrotic processes. Taking into consideration our results, we conclude that the carbamates evaluated in this study have a moderate hepatotoxic effect that is manifested several days post-treatment. Further studies are needed to assess the chronic and sub-chronic damage on the liver caused by these carbamates in order to fully determine their toxicity.

The kidneys are organs that purify toxic substances and are frequently affected by the toxic action of some compounds. An increase in the plasma levels of creatinine and the presence of lesions that suggest coagulative necrosis in the renal cortex of rats treated with high doses of the carbamates in this study indicate a slight nephrotoxic effect.

One of the most important factors in determining the toxicity of a drug is its rate of absorption, which generally varies according to the administration pathway.

In this study, although the plasma levels of the carbamates were not measured, higher amounts of toxic effects (clinical signs, lesions and functional alterations) were observed in rats treated orally than in those treated dermally. This finding suggests that these compounds are well absorbed in the digestive tract but have limited absorption through the skin. Thus, it is important that future studies evaluate the absorption, distribution, metabolism and excretion of these carbamates.

The results obtained in this study indicate that both carbamates administered orally in a single dose are low hazard. Nevertheless, there are signs of toxicity at the higher dosages, which will allow us to determine the dosage range that can be used in future chronic and sub-chronic toxicity studies. The probable administration pathway of these compounds for tick control is dermal. The low dermal acute toxicity and the absence of erythema, edema or corrosion on the skin of rats that were exposed to these carbamates make it possible to test them on bovines in order to evaluate their efficacy in the control of the cattle tick *R. microplus*.

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## TABLES AND FIGURES

Table 1. Chemical structures and molecular weights of the evaluated carbamates.

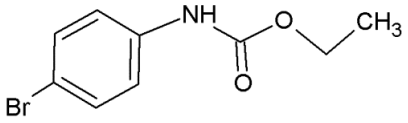
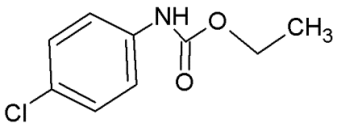
| Carbamate identification code | Chemical structure | Molecular weight (g/mol) |
|---|---|---|
| LQM 919 |  Ethyl (4-bromophenyl)carbamate | 244 |
| LQM 996 |  Ethyl (4-chlorophenyl)carbamate | 199.63 |

Table 2. Mortality of rats treated and untreated with two new carbamates administrated by oral or dermal pathway.

| TREATMENT | | LQM919 | LQM 996 | Control (corn oil or water) | Control (corn oil or water + DMSO) |
|---|---|---|---|---|---|
| Administration Pathway | Dose (mg/kg) | MORTALITY (n=5) | | | |
| Oral | 0 | -- | -- | 0 | 0 |
| | 5 | 0 | 0 | -- | -- |
| | 50 | 0 | 0 | -- | -- |
| | 300 | 1 | 0 | -- | -- |
| | 2000 | 4 | 5 | -- | -- |
| Dermal | 0 | -- | -- | 0 | 0 |
| | 500 | 0 | 0 | -- | -- |
| | 2000 | 0 | 0 | -- | -- |
| | 5000 | 0 | 0 | -- | -- |

Table 3. Clinical signs observed in rats orally dosed with carbamates LQM 919 or LQM 996. p.t. = post-treatment.

| Carbamate | Dose (mg/kg b.wt.) | Number of rats (n=5) | Clinical signs | Onset of clinical signs (hours p.t.) | Recovery time (hours p.t.) | Time of death or euthanasia (hours p.t.) |
|---|---|---|---|---|---|---|
| LQM 919 | 300 | 1 | hypotensive shock | 0.016 | | death 0.016 |
| | | 4 | mild depression in unprovoked and provoked behavior | 1 | 8 to 24 | |
| | 2000 | 1 | weakness and prostration | 1 | 8 to 24 | |
| | | 1 | weakness, postration and hypothermia | 1 | | death 3 |
| | | 3 | | 1 | | euthanasia 8 to 22 |
| LQM 996 | 300 | 5 | mild depression in unprovoked and provoked behavior | 1 | 8 to 24 | |
| | 2000 | 3 | weakness, postration and hypothermia | 1 | | euthanasia 9 |
| | | 2 | | 1 | | death 5 |

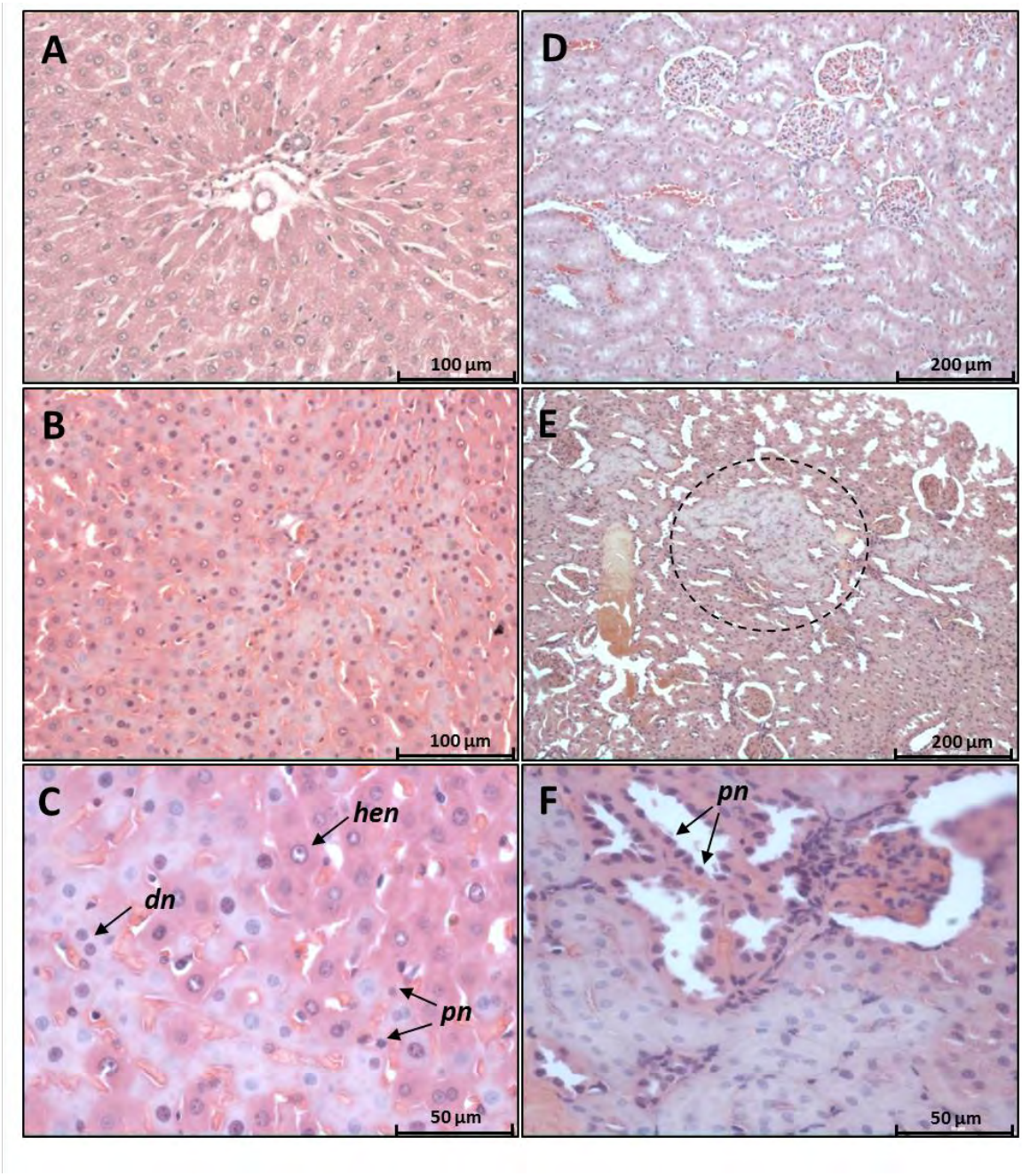



Figure 1. Histopathological findings in rats with oral exposure to carbamate LQM 919. A, Liver section (20x) of an unexposed rat (control). B, Liver section (20x) of a rat exposed to 2000 mg/kg of carbamate that shows a degenerated area. C, Previous image magnification (40x) that shows highly euchromatic nuclei (*hen*), hepatocytes with two nuclei (*dn*) and pyknotic nuclei (*pn*). D, Kidney section (10x) of an unexposed rat (control). E, Kidney section (10x) of a rat exposed to 2000 mg/kg of carbamate that shows hyaline degenerated foci (discontinous circle) in the renal cortex. F, Previous image magnification (40x) that shows hyaline degeneration and pyknotic nuclei (coagulative necrosis).

## REFERENCES

Angeles E, Mart nez P, Keller J, Martínez R, Rubio M, Ram rez G, Castillo R, López-Castañares R, Jiménez E. Ethyl and methylphenylcarbamates as antihelmintic agents: theoretical study for predicting their biological activity by PM3. J Mol Struct: THEOCHEM 2000; 504: 141-170.

Baron RL. Carbamate insecticides, In: Hayes WJ, Jr, Laws ER, Jr, editors. Handbook of Pesticide Toxicology, Academic Press; 1991. pp 3-6.

Bartels H, Bohmer M, Heierli C. Serum creatinine determination without protein precipitation. Clin Chim Acta Int J Clin Chem 1972; 37: 193-197.

Coban YK, Inanc F. Low-volume tumescent liposuction does not change plasma malondialdehyde levels: a preliminary study. Aesth Plast Surg 2004; 28: 321-323.

Dayan AD. Albendazole, mebendazole and praziquantel. Review of non-clinical toxicity and pharmacokinetics. Acta Trop 2003; 86: 141-159.

Draize JH, Alvarez E, Whitesell MF. Toxicity and primary irritation of some chemical compounds following oral administration and skin application. Fed Proc 1946; 5: 174.

Ellman GL, Courtney KD, Andres V, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961; 7: 88-95.

Foil LD, Coleman P, Eisler M, Fragoso-Sanchez H, Garcia-Vazquez Z, Guerrero FD, Jonsson NN, Langstaff IG, Li AY, Machila N, Miller RJ, Morton J, Pruett JH, Torr S. Factors that influence the prevalence of acaricide resistance and tick-borne diseases. Vet Parasitol 2004; 125: 163-181.

Gornall AG, Bardawill CJ, David MM. Determination of serum proteins by means of the biuret reaction. J Biol Chem 1949; 177: 751-766.

Irie M, Sohda T, Iwata K, Kunimoto H, Fukunaga A, Kuno S, Yotsumoto K, Sakurai K, Iwashita H, Hirano G, Ueda SI, Yokoyama K, Morihara D, Nishizawa S, Anan A, Takeyama Y, Sakamoto M, Shakado S, Sakisaka S. Levels of the oxidative stress marker gamma-glutamyltranspeptidase at different stages of nonalcoholic fatty liver disease. J Int Med Res 2012; 40: 924-933.

Kegley SE, Hill BR, Orme S, Choi AH. PAN Pesticide Database, Pesticide Action Network, North America (San Francisco, CA, 2011), http://www.pesticideinfo.org.

Lykkesfeldt J. Determination of malondialdehyde as dithiobarbituric acid adduct in biological samples by HPLC with fluorescence detection: comparison with ultraviolet-visible spectrophotometry. Clin Chem 2001; 47:1725-1727.

Morton DB, Griffiths PH. Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. Vet Rec 1985; 116: 431-436.

NOM-062-ZOO-1999, Norma Oficial Mexicana. Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio.

OECD. Guideline for testing of chemicals, 420 Acute Oral Toxicity-Fixed Dose Procedure, 2001.

Oliveira MC, Oliveira-Sequeira TC, Araujo JP, Amarante AF, Oliveira HN. *Babesia spp*. infection in *Boophilus microplus* engorged females and eggs in Sao Paulo State, Brazil. Vet Parasitol 2005; 130: 61-67.

Pérez-González IE, Prado-Ochoa MG, Muñoz-Guzmán MA, Vázquez-Valadez VH, Velázquez-Sánchez AM, Avila-Suárez BL, Cuenca-Verde C, Angeles, E, Alba-Hurtado F. Effect of new ethyl and methyl carbamates on *Rhipicephalus microplus* larvae and adult ticks resistant to conventional ixodicides. Vet Parasitol 2013; http://dx.doi.org/10.1016/j.vetpar.2013.07.042

Prado-Ochoa MG, Muñoz-Guzmán MA, Abrego-Reyes VH, Velázquez-Sánchez AM, Lara-Rocha M, Cuenca-Verde C, Angeles E, Alba-Hurtado F. Effect of new ethyl and methyl carbamates on biological parameters and reproduction of the cattle tick *Rhipicephalus microplus*. Vet Parasitol 2013a; 194: 49-57.

Prado-Ochoa MG, Ramírez-Noguera P, Díaz-Torres R, Vázquez-Valadez VH, Velázquez-Sánchez AM, Angeles E, Muñoz-Guzmán M, Alba-Hurtado F. Effect of new ethyl carbamates on acetylcholinesterase of *Rhipicephalus microplus* larvae. 31st World Veterinary Congress, Prague, Czech Rep. 2013b.

Ravuri C, Svineng G, Huseby NE. Differential regulation of gamma-glutamyltransferase and glutamate cysteine ligase expression after mitochondrial uncoupling: gamma-glutamyltransferase is regulated in an Nrf2- and NFkappaB-independent manner. Free Radic Res 2013; 47: 394-403.

Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am J Clin Pathol 1957; 28: 56-63.

Soberanes-Céspedes N, Rosario Cruz C, Garcia Vazquez Z. Variabilidad en la actividad general de esterasas de la garrapata *Boophilus microplus* y su relación con la resistencia a organofosforados. Tec Pecu Mex 2005; 43: 239-246.

Szasz G. A kinetic photometric method for serum gamma-glutamyl transpeptidase. Clin Chem 1969; 15: 124-136.

U.S. Environmental Protection Agency, Reregistration Eligibility Decision Propoxur. EPA 738-97-009. 1997.

U.S. Environmental Protection Agency. Revised EFED Risk Assessment of Carbaryl in Support of the Reregistration Eligibility Decision (RED). 2003; www.epa.gov/espp/effects/carb-riskass.pdf

UN – United Nations. Globally harmonized system of classification and labeling of chemicals. New York: United Nations. 2011.

Westgard JO, Poquette MA. Determination of serum albumin with the "SMA 12-60" by a bromcresol green dye-binding method. Clin Chem 1972;18: 647-653.

Wooton EJ. Micro-Analysis in Medical Biochemestry. Churchill; 1964.

# Capítulo 9

## SUBCHRONIC TOXICITY STUDY IN RATS OF TWO NEW ETHYL-CARBAMATES WITH ACARICIDAL ACTIVITY

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**ABSTRACT**

Female and male Wistar rats were used to determine the sub-chronic oral toxicity of two new ethyl-carbamates with ixodicide activity (ethyl-4-bromphenyl-carbamate and ethyl-4-chlorphenyl-carbamate). Evaluated carbamates were administered in drinking water (12.5, 25 and 50 mg/kg/day) for 90 days. Exposure to the evaluated carbamates did not cause mortality or clinical signs and neither did it affect feed consumption or weight gain. In contrast, exposure produced alterations to water consumption, hematocrit, percentage of reticulocytes, plasma proteins, some biochemical parameters (aspartate aminotransferase, gamma-glutamyl transpeptidase, cholinesterase and creatinine), thiobarbituric acid reactive substances and relative weight of the spleen. Histologically, slight pathological alterations were found in the liver which was consistent with the observed biochemical alterations. The non-observed-adverse-effect-level (NOAEL) of the evaluated carbamates was 12.5 mg/kg/day in both female and male rats. The low severity and reversibility of the majority of the observed alterations suggest that the evaluated carbamates have low sub-chronic toxicity.

Keywords: Subchronic toxicity, rats, ethyl carbamates, acaricide, *Rhipicephalus microplus*

## INTRODUCTION

Carbamates are relatively simple molecules characterized by being carbamic acid esters. These have been used as pesticides in agriculture, as drug therapy in humans (Alzheimer's disease, myasthenia gravis, glaucoma and as prophylaxis in organophosphate gas poisoning), and in veterinary medicine as antiparasitic drugs (Gupta, 2006). The new carbamates (designed and synthesized in FES-Cuautitlan-UNAM) ethyl-4-bromophenyl-carbamate (LQM 919) and ethyl-4-chlorophenyl-carbamate (LQM 996) inhibit oogenesis and damage ovary cells therefore affecting the reproduction of the cattle tick *Riphicephalus microplus* both in strains that are susceptible and resistant to commercial ixodicides used in Mexico (Pérez-González et al., 2013; Prado-Ochoa et al., 2013b; Prado-Ochoa et al., 2013c). As such, they have been suggested as an option for tick control.

Toxicity of carbamates is highly variable; some such as Aldicarb (oral $LD_{50}$ 0.3 to 0.9 mg/kg), carbofuran (oral $LD_{50}$ 8 mg/kg) and Carbaryl (oral $LD_{50}$ 12.5 mg/kg) are highly toxic (EPA, 2003). Others such as propoxur (oral $LD_{50}$ 68 to 94 mg/kg and dermal $LD_{50}$ >2000 mg/kg) are considered to have mid-toxicity (EPA, 1997), while benzimidazoles are considered as low toxicity. Albendazol has an $LD_{50}$ of 1320 to 2400 mg/kg and mebendazol has an oral $LD_{50}$ from 715 to 1434 mg/kg (Dayan, 2003; Kegley et al., 2009). Our group has demonstrated that carbamates LQM 919 and LQM 996 are orally low hazard ($LD_{50}$ 300-2000 mg/kg) and have a low dermal acute toxicity ($LD_{50}$ >5000 mg/kg), according to the Globally Harmonized System (GHS) of Classification and Labeling of Chemical Substances. Oral administration of high dosages in rats caused nervous system manifestations and liver damage, while dermal administration of up to 5000 mg/kg of both carbamates did not produce clinical manifestations or liver or skin alterations (Prado-Ochoa et al., 2013a).

Aspersion or immersion baths are the application pathways for the majority of the compounds used for controlling ticks in cattle. These methods have also been suggested for the application of these new carbamates (Prado-Ochoa et al., 2013b). Continuous baths of cattle cause the accumulation of the compounds in the environment and prolonged contact with animals and humans that handle them. Since carbamates LQM 919 and LQM 996 are newly synthesized their mid- and long-term toxicity effects are unknown. Taking this into consideration, the purpose of this work was to evaluate the sub-chronic toxicity caused by the oral administration of these carbamates in rats.

## MATERIALS AND METHODS

### Animals

Wistar rats between 7 and 8 weeks old (41 females and 41 males) that were clinically healthy weighing between 170 to 200 g were used. Rats were housed in polypropylene cages with same-gender companions. The environmental temperature was kept at 22 ± 2°C, with 30-70% relative humidity and the photoperiod, was maintained at 12 x 12 hours light-darkness. Rats were fed with commercial feed and water *ad libitum*. This study was approved by the Internal Committee for Care of Experimental Animals of the Postgraduate Program of Animal Production and Health (UNAM, Mexico).

### Evaluated carbamates

The carbamates used in this study were designed and synthesized at the National Autonomous University of Mexico, using a benzimidazole molecule as the structural base. They were synthesized by reacting aryl- and alkylamines with sodium hydride and benzene diethylcarbonate and were then purified using column chromatography; next, the products were recrystallized. They were structurally characterized by interpretation of their infrared spectra, hydrogen and carbon-13

nuclear magnetic resonance and mass spectrometry (Angeles et al., 2000). The chemical structure, nomenclature, molecular weight and identification codes of the evaluated carbamates can be seen in Table 1.

**Experimental design**

Rats were randomly assigned into 9 groups. The number of rats and treatment of each group is shown in Table 2. The selected dosages were based on previous acute oral toxicity studies (Prado-Ochoa et al., 2013a).

Carbamates were administered in drinking water daily for 90 days. The concentrations of carbamates were adjusted every 7 days taking into account the weight of the rats and the average daily water consumption. At the end of the 90 days, the rats in groups 1, 2, 3, 4, 6, 7 and 8 were euthanized using humane procedures (NOM-062-ZOO-1999). In order to observe the reversibility of clinical signs or lesions, groups 5 and 9 (satellite groups) remained under observation 21 additional days without treatment, after which, they were also euthanized.

After euthanasia, all rats were necropsied and the pathological findings were recorded. Liver, kidneys, brain and spleen were weighed and their relative weight was calculated (% of body weight). Samples were taken from the heart, liver, stomach, intestine, kidney, brain, spleen, testicles, ovaries and uterus for histopathology. Furthermore, blood and liver samples were collected for biochemical analysis.

**Clinical observations**

Rats were checked-on twice daily beginning 7 days before exposure and during the 90 days of exposure to the carbamates. Satellite groups were observed 21 additional days. The skin, mucosa, eyes, respiratory frequency, nasal secretions, salivation, presence of tremors, convulsions, changes in the activity level, posture, gait, sense response to stimuli and strange behavior were systematically checked

according to the table proposed by Morton and Griffiths (1985). The consumption of food and water was measured daily, while the weight of the rats was checked once a week.

**Histopathology examination**

For histopathological examinations, portions of organs were fixed in 4% paraformaldehyde and embedded in paraffin. Embedded organs were cut into 5 µm thick sections and the sections were stained with hematoxylin-eosin. Sections were observed under optical microscope.

**Biochemical tests**

Plasma was obtained from the blood samples and frozen at -80 ºC until their processing. The plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and gamma-glutamyl transferase (GGT) were measured using the methods described by Reitman and Frankel (1957), Wooton (1964) and Szasz (1969). The total protein, albumin and creatinine concentrations in plasma were determined using the methods of Westgard and Poquette (1972), Gornal (1949) and Bartels (1972). The aforementioned determinations were carried out using Kits from BioSystems®. The value obtained for albumin was subtracted from amount of total proteins in order to calculate the concentration of globulins in plasma. The relationship between albumin and globulin (A/G) was calculated by dividing the concentration of albumin by the concentration of globulin. The cholinesterase activity (CHE) was determined using Ellman's (1961) colorimetric method using a Kit from Wiener lab®.

**TBARS quantification**

Samples 0.5 $cm^3$ in size were collected from the liver of all euthanized rats in order to estimate the oxidation-reduction state in the liver of the rats exposed to the carbamates in this study. These samples were submerged in a buffer solution (PBS

pH 7.2, sodium azide 15mM, PMSF 1mM, Triton X-100 0.1% and EDTA 5 mM) and frozen in liquid nitrogen until their processing. Tissues were thawed on ice, the original buffer solution discarded, new buffer solution added, mechanically homogenized, sonicated (3 pulses, 10 seconds, 50% amplitude) and finally centrifuged (13000 g, 7º to 4 °C). An equivalent volume of 2.5% perchloric acid was added to the supernatant (40 µL) and incubated for 10 minutes at ambient temperature. Afterwards, these were centrifuged at 13000 g for 10 minutes at 4º C, the supernatant made to react with 0.067% thiobarbituric acid (TBA) at 90º C for 30 minutes (Coban and Inanc, 2004). TBARS content of the samples was quantified using a standard curve generated from known concentrations of malondialdehyde (MDA) at 532 nm (Lykkesfeldt, 2001). TBARS concentrations were expressed in nmol/mg of proteins.

**Statistical analysis**

The data on weight gain, relative organ weight, food and water consumption, haematocrit, percentage of reticulocytes in blood, plasma levels of AST, GGT, LDH, ALT, CHE and creatinine, as well as TBARS concentrations in liver were analyzed using a one-way ANOVA, with differences between means established by Fisher's *post hoc* analysis (minimum significant difference) using a minimum confidence level of 95%.

**RESULTS**

**Clinical signs**

Exposure to carbamates LQM 919 or LQM 996 did not cause mortality in any of the rats exposed to them. No clinical signs or adverse effects associated with toxicity were observed in the rats exposed to the carbamates throughout the 90 days of exposure or during the additional observation time of the satellite groups.

**Food and water consumption**

Average daily food and water consumptions are shown in Figure 1. The exposure to carbamates LQM 919 or LQM 996 did not decrease feed consumption in rats in any of the treated groups when compared to the rats in the control groups ($p>0.05$). Females of the treated groups with both carbamates, including the rats in the satellite groups, had a reduced ($p<0.05$) average daily water consumption during the period of exposure to carbamates when compared to unexposed females. Rats in the satellite groups did not show differences in the daily water consumption in regards to the control groups during the 21 days after exposure ($p>0.05$).

**Body weight and organ weight behavior**

Average weight gains of rats exposed to carbamates are shown in Figure 2. The administration of both carbamates did not alter the weight gain in rats from any of the experimental groups when compared to the rats in the corresponding control groups ($P>0.05$).

Relative weights of liver, kidney, brain and spleen of the various groups are shown in Table 3. The relative weigh of the spleen ($P<0.05$) was greater in female and male rats exposed to 25 and 50 mg/kg of LQM 919 and with all dosages of LQM 996 when compared to rats of the corresponding control groups. No statistical differences ($p>0.05$) were found between the relative weigh of the spleen in rats of satellite groups when compared to rats in the control group. No statistical differences ($p>0.05$) were observed in the relative weight of liver, kidney and brain of the various groups in regards to the controls.

**Biochemical and hematological parameters**

Tables 4 and 5 show the biochemical parameters measured from the plasma obtained from rats exposed to the evaluated carbamates. Males treated with 25 mg/kg of LQM 919 had a higher plasma concentration of creatinine and total

proteins, albeit a lower concentration of CHE than untreated males ($p<0.05$). Females exposed to 50 mg/kg of LQM 919 had a higher plasma concentration ($p<0.05$) of creatinine when compared to unexposed rats. Males treated with 25 and 50 mg/kg of LQM 996 had a higher plasma concentration of GGT ($p<0.05$) when compared to unexposed males. No statistical differences ($p>0.05$) were observed among the remainder of the plasma parameters between the treated and untreated rat groups.

Males exposed to 50 mg/kg of both carbamates had a higher hematocrit ($p<0.05$) when compared to unexposed groups. The exposure of female and male rats to the evaluated carbamates caused an increase in circulating reticulocytes ($p<0.05$) when compared to rats in the control groups. Satellite groups (21 days after the final exposure) did not show statistical differences when compared to rats in the control group.

**Histopathology**

Histopathological changes produced in the rats in the groups treated with 25 and 50 mg/kg of both carbamates, including the satellite groups, were similar. Moderate albuminous degeneration, slight periacinar vacuolar degeneration, moderate diffuse congestions and some hepatocytes with highly euchromatic nuclei or grayish cytoplasm could be observed in the liver. The kidneys showed slight interstitial cell infiltrate. Furthermore, hemosiderin deposits could be observed in the spleen, kidneys and ovaries (Figure 3).

**TBARS**

Figure 3 shows the TBARS concentrations in liver of rats exposed to the evaluated carbamates. The rats exposed to LQM 996 did not show statistical differences ($p>0.05$) in TBARS concentration when compared to the control group. Induction of TBARS when the rats were exposed to LQM 919 was significantly different to the control group both for females and males at the maximum evaluated

dosage (50 mg/kg/day). Even when there was no significant dosage-response effect in both sexes, a trend could be seen at the range of dosages evaluated (Figure 4).

## DISCUSSION

It has been demonstrated *in vitro* that carbamates LQM 996 and LQM 919 inhibit the reproduction of *R. microplus* susceptible and resistant strains to conventional acaricides such as organophosphates, pyrethroids, and amidines (Pérez-González et al., 2013; Prado-Ochoa et al., 2013b), which demonstrates their potential use in tick control. The results obtained in this study demonstrate that sub-chronic exposure to the evaluated carbamates produce slight pathological alterations in rats.

Sub-chronic oral toxicity studies evaluate the adverse effects caused by prolonged exposure of animals to a substance and provide information on their negative effects upon target organs as well as their cumulative effects. Furthermore, these studies determine the dosage at which no observable adverse effects are present. In this study the exposure to carbamates LQM 919 or LQM 996 was well tolerated and the absence of deaths or clinical signs of toxicity related to exposure are an indicator of the relative harmless nature of the evaluated carbamates over prolonged exposure.

It has been proposed that compounds with toxic potential impact the consumption of food, metabolic processes and in consequence on weight gain. Studies that have evaluated long-term effects of a compound have considered a reduction greater than 10% in weight gain to be detrimental to animals (Homburger, 1989). No differences were observed in food consumption and weight gain between animals exposed and those unexposed to carbamates in this study. In contrast, weight gain of female and male rats of the various groups showed continuous increase suggesting that there were no severe alterations of the metabolic processes of rats exposed to the carbamates.

Adequate water consumption is essential for the normal physiological processes of an animal. It has been observed that some substances can modify its consumption and therefore have an effect on their metabolism (Hainsworth et al., 1968). We found that female rats exposed to the evaluated carbamates decreased their water consumption when compared to the control groups. This could be due to the flavor of carbamates since females of the satellite groups regained their normal water consumption level when the exposure to carbamates was terminated. It has been shown that male and female rats have differences in their taste response and/or taste perception (Curtis et al., 2004), which could explain the difference in the response to water consumption observed in this study. This decrease in water intake was not associated to dehydration signs.

Detoxification processes are mainly carried out in the liver; this organ together with the kidney has the greatest exposure to xenobiotics and/or their metabolites. The susceptibility of liver and renal tissues to stress due to pesticide exposure depends on the general balance between the degree of oxidative stress and the antioxidant capacity (Khan et al., 2005). Serum enzymes including ALT, AST, LDH and GGT are mainly used for assessing liver damage. Our results show that exposure to the evaluated carbamates caused an elevation of the AST and GGT enzymes in the plasma from males, while only GGT in that of females at the maximum dosage evaluated. These results, together with the slight histopathology alterations observed and the moderate TBARS increase (oxidative damage) show that these carbamates are slightly hepatotoxic. Other carbamates (carbofuran and propoxur), as well as some organophosphates (chlorpyrifos), that are commercially used as acaricides cause similar liver alterations (Brkic et al., 2008; Eraslan et al., 2009; Mansour and Mossa, 2010). The results from the GGT analysis are interesting (Table 5) since the enzyme has been shown to have important functions in the control of cell homeostasis associated to oxidative stress (Irie et al., 2012). In this study, GGT increased significantly in male rats exposed to LQM 996 at a 25 and 50

mg/kg/day dosage when compared to control rats; the effect does not correlate with the TBARS estimates in which there were no significant differences when compared to controls. Nevertheless, previous studies carried out in our group showed that the same carbamate when administered orally to male rats at a 5 mg/kg/day dose in an acute exposure design was capable of inducing a significant increase in the amount of TBARS and GGT (Prado-Ochoa et al., 2013a). If we take into consideration the role of the cell oxidative stress modulator, and specifically its function in the reduction of oxidized glutathion regulating its levels (main cell antioxidant) (Filomeni et al., 2002), as well as the induction associated to the sub-chronic exposure to LQM 919 we consider that in rats exposed to this carbamate, the GGT could have been capable of performing redox modulating actions (Figure 3). Therefore, if the oxidative stress increases, the compensatory effects of GGT could be at risk. Based upon the results obtained we suggest further studies on the mechanisms of action involved in the exposure to carbamates LQM 919 and LQM 996, and their role in the modulation of cell oxidative stress.

It has been observed that the susceptibility to a xenobiotic can vary depending on the sex, The differences in liver susceptibility to the evaluated carbamates between males and females observed in this study could be due to a higher capacity of males for liver metabolism and breakdown of chemicals (greater quantity of cytochrome P450) making them more or less susceptible to toxicity of a compound depending on their taking a bioactivation pathway or a detoxification one (Franklin and Yost, 2003). Further studies on biotransformation of these carbamates should allow the determination of their metabolism pathway. These results allow a relationship to be made between the evidence obtained regarding their observed biological effects and the mechanisms of action associated to their *in vivo* and *in vitro* exposure taking into consideration the interest on its use as an ixodicide.

It has been observed that some pesticide products have hemolytic activities. In this study we did not directly evaluate such activity. Nevertheless, some findings

such as splenomegaly and the presence of hemosiderin in various organs (spleen, kidney and ovary) suggest an increase in the rate of erythrocytes destruction. Also, the increase of reticulocytes and haematocrit in rats in the groups treated with the highest exposure dosages could be related to a compensatory response of the bone marrow.

The evaluated carbamates have an inhibitory effect on the development of the ovary cells in *R. microplus* Ticks (Prado-Ochoa et al., 2013c) which prompts the study of the effects of these carbamates on the ovary of mammals. The histological review of the ovary in rats exposed to both carbamates did not reveal apparent pathological changes; the single deposit of hemosiderin in the organs of some rats was considered to be unrelated to the direct damage of this organ. Future studies would allow the evaluation of the effects on mammal reproduction.

When a new chemical is evaluated it is important to determine the non-observed-adverse-effect level (NOAEL) in order to assess the risks associated to its use. Based on the results of this study, the evaluated carbamates show a NOAEL level of 12.5 mg/kg for female and male Wistar rats. Nevertheless, the results obtained at dosages of 25 and 50 mg/kg of the evaluated carbamates suggest caution and the need for complementary studies that evaluate their *in vivo* and *in vitro* cytotoxic and genotoxic potential, as well as the repercussions to the environment that the use of carbamates LQM 919 and LQM 996 could entail.

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## TABLES AND FIGURES

Table 1. Chemical structures and molecular weights of the evaluated carbamates.

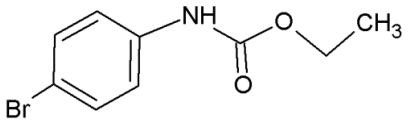
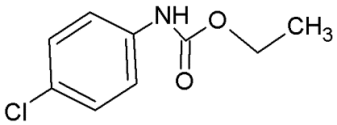
| Carbamate identification code | Chemical structure | Molecular weight (g/mol) |
|---|---|---|
| LQM 919 |  Ethyl (4-bromophenyl)carbamate | 244 |
| LQM 996 |  Ethyl (4-chlorophenyl)carbamate | 199.63 |

Table 2. Experimental groups

| Group | Treatment | Rats: number and sex | Treatment days /observation days after treatment |
|---|---|---|---|
| 1 | Control group (0.1% DMSO) | 5 males and 5 females | 90/0 |
| 2 | LQM 919 (12.5 mg/kg/day) | 5 males and 5 females | 90/0 |
| 3 | LQM 919 (25 mg/kg/day) | 5 males and 5 females | 90/0 |
| 4 | LQM 919 (50 mg/kg.day) | 5 males and 5 females | 90/0 |
| 5 | LQM 919 (25 mg/kg/day), satellite group | 3 males and 3 females | 90/21 |
| 6 | LQM 996 (12.5 mg/kg/day) | 5 males and 5 females | 90/0 |
| 7 | LQM 996 (25 mg/kg.b/day) | 5 males and 5 females | 90/0 |
| 8 | LQM 996 (50 mg/kg/day) | 5 males and 5 females | 90/0 |
| 9 | LQM 996 (25 mg/kg/day), satellite group | 3 males and 3 females | 90/21 |

Table 3. Weight of organs relative to body weight (%) of female and male Wistar rats subchronically exposed with LQM 919 or LQM 996.

| TREATMENT | liver | | kidney | | brain | | spleen | |
|---|---|---|---|---|---|---|---|---|
| | females | males | females | males | females | males | females | males |
| **LQM 919 (50 mg/kg/day)** | 3.43 (± 0.18)[a] | 3.22 (± 0.22)[a] | 0.36 (± 0.02)[a] | 0.35 (± 0.04)[a] | 0.75 (± 0.07)[a] | 0.49 (± 0.04)[a] | 0.38(± 0.08)[a] | 0.28 (± 0.04)[a] |
| **LQM 919 (25 mg/kg/day)** | 3.86 (± 0.47)[a] | 3.08 (± 0.03)[a] | 0.33 (± 0.01)[a] | 0.33 (± 0.01)[a] | 0.70 (± 0.17)[a] | 0.41 (± 0.14)[a] | 0.51(± 0.17)[b] | 0.41 (± 0.14)[b] |
| **LQM 919 (12.5 mg/kg/day)** | 3.35 (± 0.33)[a] | 3.54 (± 0.37)[a] | 0.35 (± 0.04)[a] | 0.33 (± 0.01)[a] | 0.65 (± 0.04)[a] | 0.52 (± 0.10)[a] | 0.35(± 0.04)[a] | 0.22 (± 0.01)[a] |
| **LQM 919 (25 mg/kg/day) S*** | 3.83 (± 0.01)[a] | 3.60 (± 0.24)[a] | 0.34 (± 0.04)[a] | 0.34 (± 0.02)[a] | 0.67 (± 0.05)[a] | 0.38 (± 0.01)[a] | 0.26 (± 0.00)[a] | 0.18 (± 0.03)[a] |
| **LQM 996 (50 mg/kg/day)** | 3.96 (± 0.34)[a] | 3.55 (± 0.24)[a] | 0.33 (± 0.02)[a] | 0.33 (± 0.01)[a] | 0.71 (± 0.08)[a] | 0.47 (± 0.04)[a] | 0.47 (± 0.06)[b] | 0.44 (± 0.08)[b] |
| **LQM 996 (25 mg/kg/day)** | 3.90 (± 0.53)[a] | 3.60 (± 0.03)[a] | 0.35 (± 0.02)[a] | 0.35 (± 0.05)[a] | 0.72 (± 0.09)[a] | 0.48 (± 0.05)[a] | 0.55 (± 0.07)[b] | 0.39 (± 0.08)[b] |
| **LQM 996 (12.5 mg/kg/day)** | 3.69 (± 0.39)[a] | 3.46 (± 0.30)[a] | 0.33 (± 0.03)[a] | 0.33 (± 0.03)[a] | 0.69 (± 0.10)[a] | 0.50 (± 0.08)[a] | 0.37 (± 0.04)[a] | 0.31 (± 0.06)[b] |
| **LQM 996 (25 mg/kg/day) S*** | 3.53 (± 0.40)[a] | 3.51 (± 0.06)[a] | 0.34 (± 0.00)[a] | 0.33 (± 0.03)[a] | 0.72 (± 0.15)[a] | 0.38 (± 0.03)[a] | 0.25 (± 0.01)[a] | 0.24 (± 0.04)[a] |
| **Control group** | 3.59 (± 0.21)[a] | 3.25 (± 0.17)[a] | 0.33 (± 0.01)[a] | 0.32 (± 0.02)[a] | 0.69 (± 0.08)[a] | 0.46 (± 0.03)[a] | 0.32 (± 0.12)[a] | 0.19 (± 0.03)[a] |

Values are mean ± SD. Different letters show statistical differences between means ($p>0.05$).

Table 4. Effect of subchronic exposure of LQM 919 on selected parameters in male and female Wistar rats.

| Group | females | | | | | males | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | control (-) | 12.5 mg/kg/day | 25 mg/kg/day | 25 mg/kg/day (S) | 50 mg/kg | Control (-) | 12.5 mg/kg/day | 25 mg/kg | 25 mg/kg/day (S) | 50 mg/kg/day |
| AST (U/L) | 238.2 (±78.13) | 259.0 (±130.4) | 152.0 (±17.12) | 125.6 (±2.81) | 202.3 (±72.55) | 195.8 (±70.16) | 166.5 (±28.12) | 276.7 (±110.3) | 358.2 (±49.30)* | 237.1 (±62.47) |
| ALT (U/L) | 61.52 (±19.55) | 51.34 (±12.73) | 68.68 (±29.46) | 76.13 (±5.54) | 59.20 (±9.31) | 60.64 (±32.21) | 76.05 (±1.96) | 63.27 (±12.37) | 45.97 (±10.45) | 73.18 (±8.11) |
| LDH (U/L) | 924.2 (±285.6) | 851.3 (±192.9) | 410.5 (±95,73) | 370.7 (±62.00) | 664.6 (±435.2) | 739.5 (±334.1) | 558.0 (±225.9) | 553.7 (±246.8) | 660.7 (±263.0) | 872.0 (±464.4) |
| GGT (U/L) | 1.71 (±2.28) | 1.08 (±1.14) | 0.77 (±0.90) | 0.38 (±0.02) | 0.18 (±0.26) | 0.34 (±0.25) | 0.51 (±0.42) | 0.43 (±0.75) | 0.25 (±0.42) | 1.33 (±0.99) |
| CHE (U/L) | 953.9 (±526.7) | 1404 (±794.8) | 982.2 (±116.6) | 1132 (±444.2) | 1088 (±137.8) | 344.3 (±111.1) | 459.0 (±138.5) | 466.2 (±96.50) | 344.6 (±33.32) | 237.28 (±140.31) |
| Creatinin (mg/dL) | 0.44 (±0.20) | 0.64 (±0.07) | 0.68 (±0.09) | 0.53 (±0.16) | 0.75 (±0.16)* | 0.54 (±0.12) | 0.65 (±0.14) | 0.65 (±0.15) | 0.46 (±0.04) | 0.74 (±0.01) |
| Total protein (g/dL) | 5.83 (±1.07) | 6.85 (±0.67) | 7.26 (±0.43) | 7.42 (±0.18) | 6.61 (±0.98) | 5.52 (±0.76) | 6.52 (±0.29) | 6.27 (±1.48) | 5.80 (±0.27) | 7.08 (±0.28)* |
| Albumin (g/dL) | 2.36 (±1.05) | 1.98 (±0.87) | 3.71 (±0.32) | 2.32 (±1.44) | 3.09 (±0.77) | 1.72 (±0.86) | 2.72 (±0.95) | 1.87 (±0.10) | 2.36 (±0.28) | 1.99 (±0.91) |
| Globulin (g/dL) | 3.47 (±0.89) | 4.87 (±1.45) | 3.55 (±0.13) | 5.09 (±1.51) | 3.52 (±1.35) | 3.80 (±0.27) | 3.81 (±1.67) | 4.40 (±1.56) | 3.45 (±0.55) | 5.09 (±0.82) |
| Reticulocytes (%) | 4.21 (±0.35) | 6.13 (±0.74) | 5.78 (±0.72) | 3.87 (±0.79) | 6.66 (±1.49)* | 3.93 (±0.77) | 5.44 (±1.07)* | 6.15 (±0.53)* | 2.75 (±0.95) | 5.87 (±0.66)* |
| Haematocrit (%) | 36.6 (±2.92) | 41.70 (±2.16) | 33.20 (±8.78) | 29.33 (±9.45) | 39.80 (±6.41) | 35.70 (±3.89) | 43.00 (±2.82) | 28.33 (±6.65) | 37.33 (±3.05) | 48.00 (±5.56)* |
| A/G | 0.74 (±0.39) | 0.49 (±0.45) | 1.04 (±0.06) | 0.54 (±0.44) | 1.00 (±0.39) | 0.46 (±0.24) | 0.76 (±0.34) | 0.48 (±0.24) | 0.70 (±0.19) | 0.43 (±0.29) |

AST=aspartate aminotransferase, ALT=alanine aminotransferase , LDH=lactate dehydrogenase , GGT= gamma-glutamyltransferase, CHE= cholinesterase, A/G= albumin and globulin, (s)= satellite group . Values are mean ± SD. * $P<0.05$ (significantly different from control).

Table 5. Effect of subchronic exposure of LQM 996 on selected parameters in male and female Wistar rats.

| | females | | | | | males | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Group** / **Parameter** | **Control (-)** | **12.5 mg/kg/day** | **25 mg/kg/day** | **25 mg/kg/day (S)** | **50 mg/kg/day** | **control (-)** | **12.5 mg/kg/day** | **25 mg/kg/day** | **25 mg/kg/day (S)** | **50 mg/kg/day** |
| **AST (U/L)** | 238.2 (±78.13) | 263.4 (±82.12) | 192.3 (±84.08) | 181.8 (±23.10) | 207.5 (±96.51) | 195.8 (±70.16) | 265.6 (±26.8) | 167.8 (±30.81) | 196.9 (±60.23) | 168.3 (±18.03) |
| **ALT (U/L)** | 61.52 (±19.55) | 90.14 (±84.82) | 44.37 (±21.49) | 83.43 (±17.10) | 52.26 (±13.00) | 60.64 (±32.21) | 74.84 (±58.61) | 38.33 (±12.79) | 72.70 (±15.67) | 63.04 (±15.46) |
| **LDH (U/L)** | 924.2 (±285.6) | 908.8 (±279.8) | 506.0 (±241.0) | 547.0 (±74.75) | 609.0 (±249.0) | 739.5 (±334.1) | 942.4 (±310.5) | 441.0 (±146.5) | 694.0 (±387.2) | 607.8 (±143.7) |
| **GGT (U/L)** | 1.71 (±2.28) | --- | 1.29 (±1.99) | 1.56 (±0.36) | 1.62 (±1.57) | 0.34 (±0.25) | 2.24 (±3.14) | 6.29 (±4.82)* | 2.10 (±1.58) | 6.59 (±5.35)* |
| **CHE (U/L)** | 953.9 (±526.7) | 1505 (±348.6) | 984.6 (±210.9) | 1754 (±838.4) | 892.8 (±317.8) | 344.3 (±111.1) | 546.4 (±308.4) | 385.0 (±71.40) | 716.5 (±488.1) | 528.0 (±94.73) |
| **Creatinin (mg/dL)** | 0.44 (±0.20) | 0.43 (±0.07) | 0.44 (±0.02) | 0.38 (±0.05) | 0.49 (±0.10) | 0.54 (±0.12) | 0.50 (±0.12) | 0.60 (±0.10) | 0.52 (±0.09) | 0.54 (±0.17) |
| **Total protein (g/dL)** | 5.83 (±1.07) | 6.92 (±1.23) | 5.88 (±1.12) | 6.98 (±0.06) | 5.93 (±0.08) | 5.52 (±0.76) | 5.96 (±0.39) | 5.95 (±0.37) | 6.26 (±0.71) | 6.38 (±0.72) |
| **Albumin (g/dL)** | 2.36 (±1.05) | 2.40 (±1.31) | 1.83 (±1.51) | 2.04 (±1.09) | 1.87 (±1.04) | 1.72 (±0.86) | 2.18 (±0.72) | 2.16 (±0.92) | 2.40 (±0.20) | 2.01 (±0.81) |
| **Globulin (g/dL)** | 3.47 (±0.89) | 4.52 (±1.53) | 4.05 (±1.36) | 4.94 (±1.12) | 4.06 (±1.01) | 3.80 (±0.27) | 3.79 (±0.84) | 3.79 (±0.55) | 3.86 (±0.84) | 4.38 (±1.43) |
| **Reticulocytes(%)** | 4.21 (±0.35) | 8.82 (±1.61)* | 10.06 (±2.15)* | 2.34 (±0.25) | 9.92 (±3.42)* | 3.93 (±0.77) | 5.23 (±1.44)* | 6.65 (±1.15)* | 3.38 (±0.37) | 6.60 (±0.97)* |
| **Haematocrit (%)** | 36.62 (±2.92) | 38.00 (±6.97) | 34.4 (±9.52) | 35.00 (±11.00) | 36.80 (±8.49) | 35.70 (±3.89) | 44.25 (±3.20)* | 37.30 (±6.41) | 41.33 (±2.51) | 43.60 (±4.72)* |
| **A/G** | 0.74 (±0.39) | 0.64 (±0.46) | 0.56 (±0.63) | 0.47 (±0.34) | 0.53 (±0.37) | 0.46 (±0.24) | 0.62 (±0.31) | 0.60 (±0.30) | 0.65 (±0.21) | 0.55 (±0.36) |

AST=aspartate aminotransferase , ALT=alanine aminotransferase , LDH=lactate dehydrogenase , GGT= gamma-glutamyltransferase, CHE= cholinesterase, A/G= albumin and globulin, (s)= satellite group. Values are mean ± SD. * $P<0.05$ (significantly different from control).

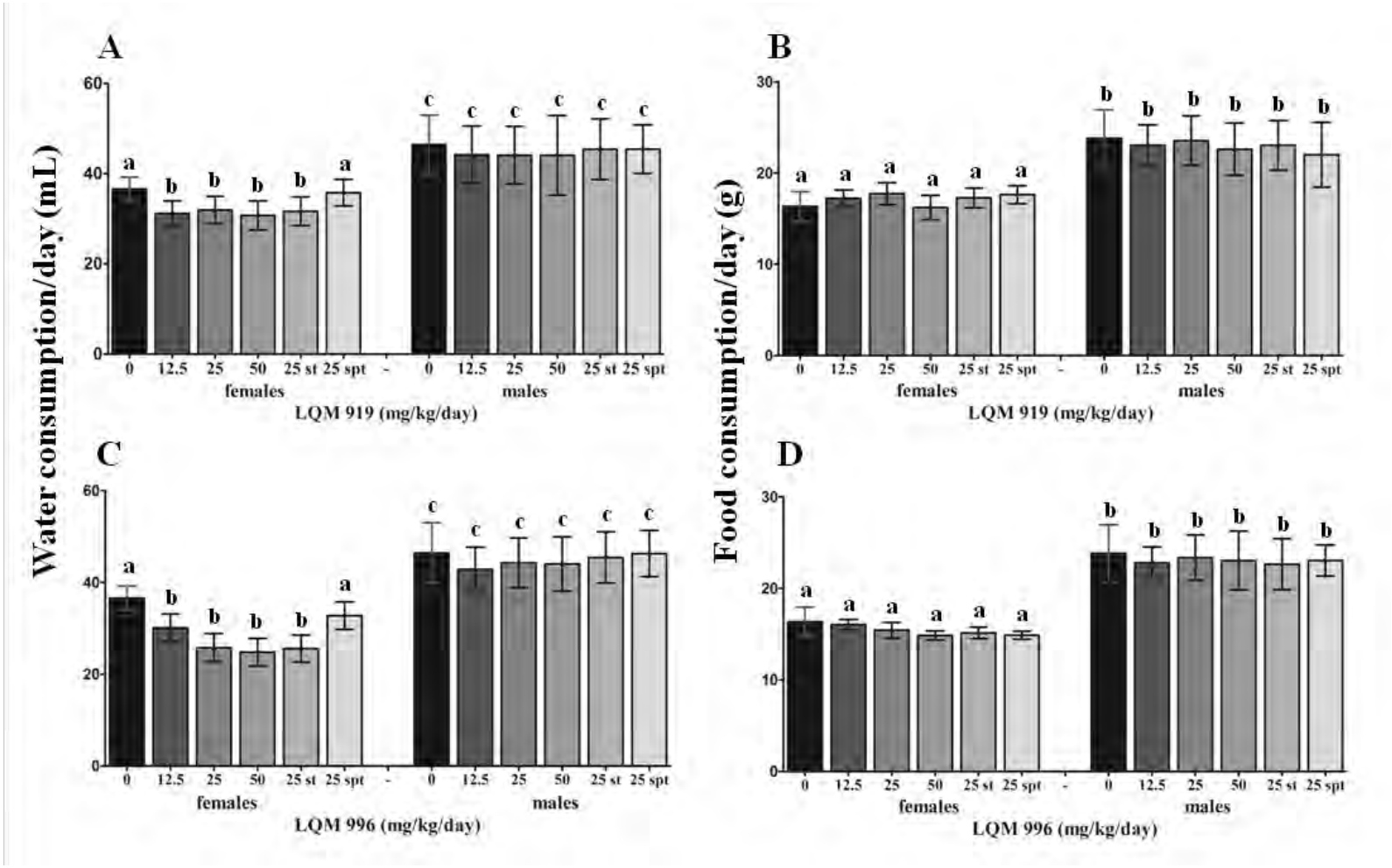



Figure 1. Food and water consumption of female and male Wistar rats exposed subchronically (90 days) with LQM 919 or LQM 996. st = satellite group during carbamate exposure (90 days), spt= satellite group during recovery time (21 days). Different letters indicate statistical differences between means ($p<0.05$).

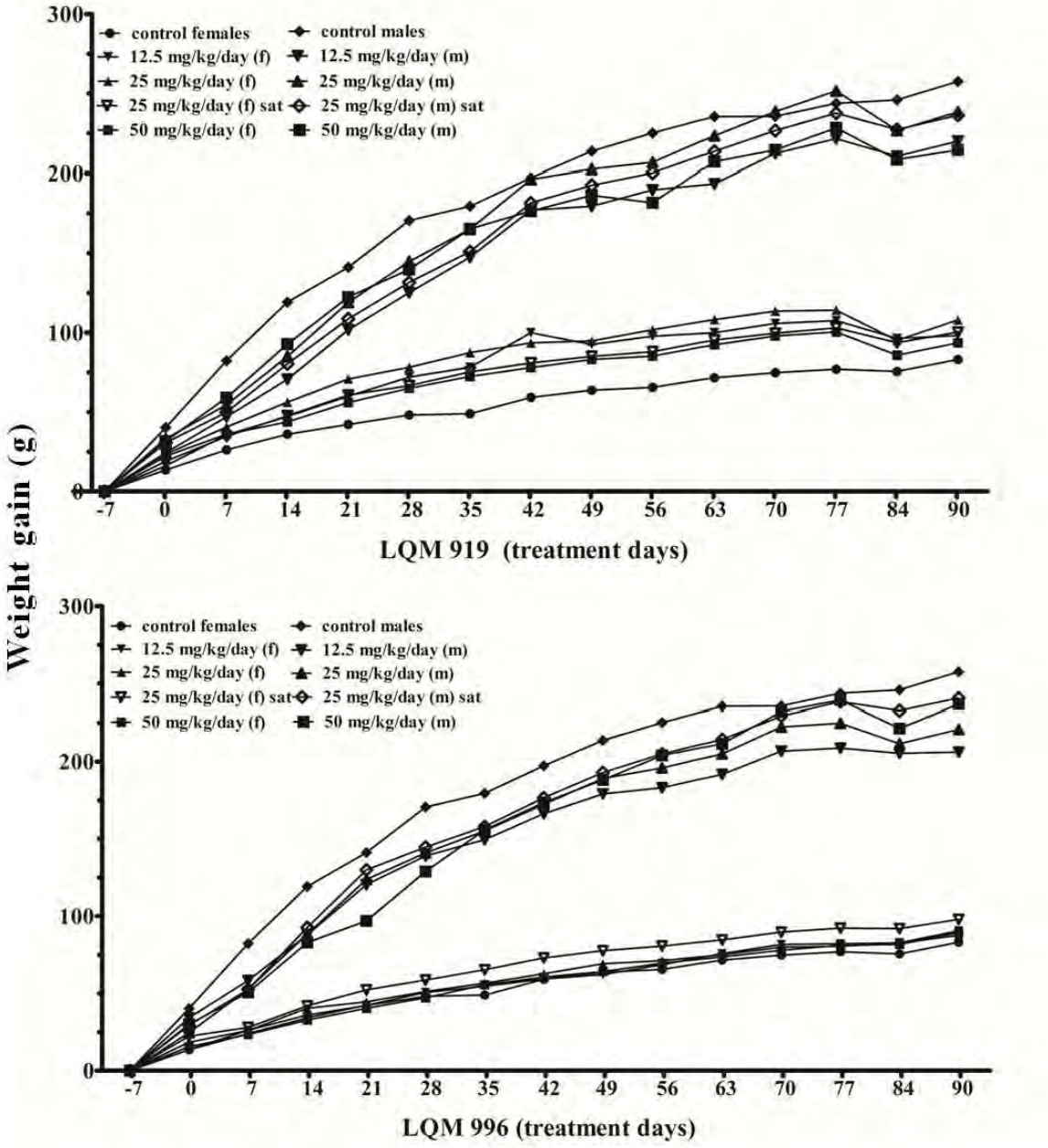



Figure 2. Weight gain of female and male Wistar rats subchronically exposed to LQM 919 or LQM 996. f= females, m=males, s= satellite group.

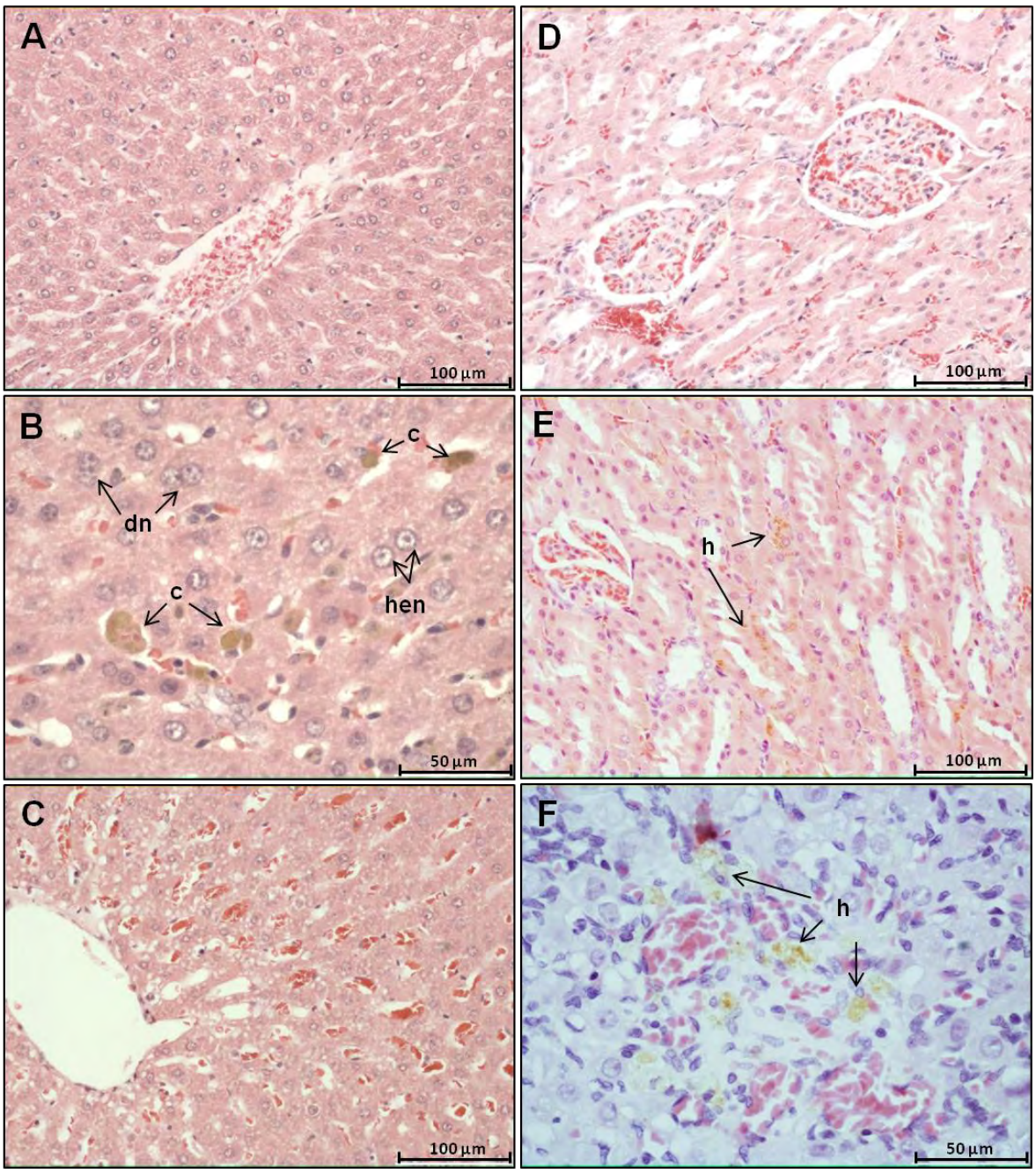



Figure 3. Histopathological findings in rats with subcronical exposure to carbamate LQM 919. A, Liver section (10x) of an unexposed rat (control). B, Liver section (20x) of a rat exposed to 50 mg/kg/day of LQM 919 that that shows highly euchromatic nuclei (hen), hepatocytes with two nuclei (dn) and colestasis (c). C, Previous image magnification (10x) shows a degenerated area. D, Kidney section (10x) of an unexposed rat (control). E, Kidney section (10x) of a rat exposed to 50 mg/kg/day of carbamate that shows hemosiderin depositions (h). F, Previous image magnification (20x) that shows hemosiderin depositions (h) in ovary of an exposed rat (50 mg/kg/day).

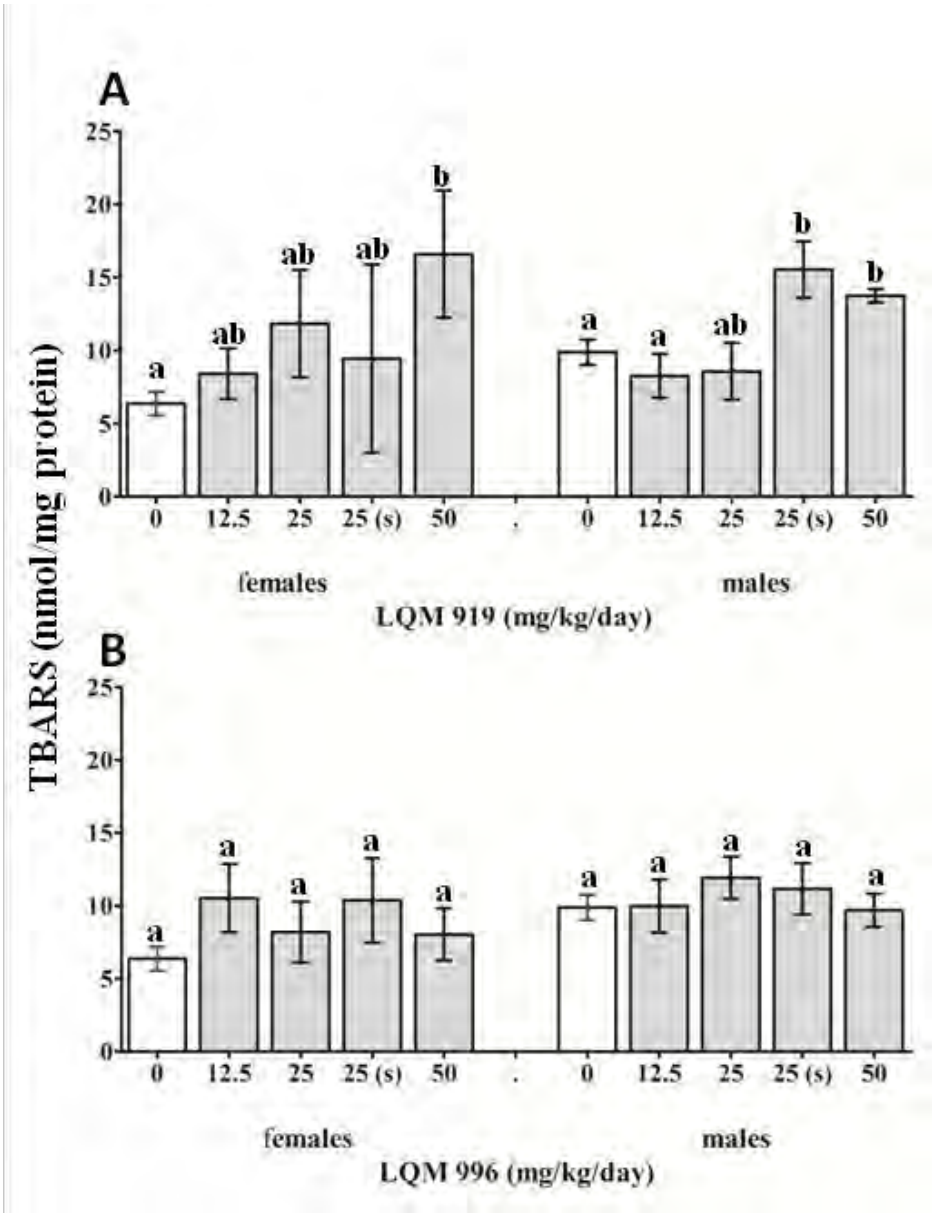



Figure 4. Thiobarbituric acid reactive substances (TBARS) in liver of female and male Wistar rats subcronically exposed to carbamates LQM 919 or LQM 996. (S)=satellite group. Different letters indicate statistical difference between means ($p<0.05$).

## REFERENCES

Angeles, E., Mart nez, P., Keller, J., Mart nez, R., Rubio, M., Ram rez, G., Castillo, R., López-Castañares, R., Jiménez, E., 2000. Ethyl and methylphenylcarbamates as antihelmintic agents: theoretical study for

predicting their biological activity by PM3. Journal of Molecular Structure: THEOCHEM 504, 141-170.

Bartels, H., Bohmer, M., Heierli, C., 1972. [Serum creatinine determination without protein precipitation]. Clin. Chim. Acta 37, 193-197.

Brkic, D.V., Vitorovic, S., Gasic, S.M., Neskovic, N.K., 2008. Carbofuran in water: Subchronic toxicity to rats. Environ. Toxicol. Pharmacol. 25, 334-341.

Coban, Y.K., Inanc, F., 2004. Low-volume tumescent liposuction does not change plasma malondialdehyde levels: a preliminary study. Aesthetic. Plast. Surg. 28, 321-323.

Curtis, K.S., Davis, L.M., Johnson, A.L., Therrien, K.L., Contreras, R.J., 2004. Sex differences in behavioral taste responses to and ingestion of sucrose and NaCl solutions by rats. Physiol. Behav. 80, 657-664.

Dayan, A.D., 2003. Albendazole, mebendazole and praziquantel. Review of non-clinical toxicity and pharmacokinetics. Acta Trop. 86, 141-159.

Ellman, G.L., Courtney, K.D., Andres, V., Jr., Feather-Stone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-95.

EPA, U.S., 1997. Reregistration Eligibility Decision Propoxur. EPA 738-97-009. Washington, DC: U.S. Environmental Protection Agency.

EPA, U.S., 2003. Environmental Protection Agency. Revised EFED Risk Assessment of Carbaryl in Support ot the Reregistration Eligibility Decision (RED). www.epa.gov/espp/effects/carb-riskass.pdf (2003).

Eraslan, G., Kanbur, M., Silici, S., Cem Liman, B., Altinordulu, S., Soyer Sarica, Z., 2009. Evaluation of protective effect of bee pollen against propoxur toxicity in rat. Ecotoxicol. Environ. Saf. 72, 931-937.

Filomeni, G., Rotilio, G., Ciriolo, M.R., 2002. Cell signalling and the glutathione redox system. Biochem. Pharmacol. 64, 1057-1064.

Franklin, M.R., Yost, G.S., 2003. Biotransformation: A Balance between Bioactivation and Detoxification, Principles of Toxicology. John Wiley & Sons, Inc., pp. 57-86.

Gornall, A.G., Bardawill, C.J., David, M.M., 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177, 751-766.

Gupta, R.C., 2006. Toxicology of organophosphate & carbamate compounds. USA. Elsevier.

Hainsworth, F.R., Stricker, E.M., Epstein, A., 1968. Water metabolism of rats in the heat: dehydration and drinking. American Journal of Physiology-Legacy Content 214, 983-989.

Homburger, F., 1989. In vivo testing in the study of toxicity and safety evaluation. A guide to general toxicology. 2nd. ed. New York: Karger.

Irie, M., Sohda, T., Iwata, K., Kunimoto, H., Fukunaga, A., Kuno, S., Yotsumoto, K., Sakurai, K., Iwashita, H., Hirano, G., Ueda, S.I., Yokoyama, K., Morihara, D., Nishizawa, S., Anan, A., Takeyama, Y., Sakamoto, M., Shakado, S., Sakisaka, S., 2012. Levels of the oxidative stress marker gamma-glutamyltranspeptidase at different stages of nonalcoholic fatty liver disease. J. Int. Med.Res. 40, 924-933.

Kegley, S., Hill, B., Orme, S., Choi, A., 2009. PAN Pesticide Database, Pesticide Action Network, North America. San Francisco, CA.

Khan, S.M., Sobti, R.C., Kataria, L., 2005. Pesticide-induced alteration in mice hepato-oxidative status and protective effects of black tea extract. Clin. Chim. Acta 358, 131-138.

Lykkesfeldt, J., 2001. Determination of malondialdehyde as dithiobarbituric acid adduct in biological samples by HPLC with fluorescence detection: comparison with ultraviolet-visible spectrophotometry. Clin. Chem. 47, 1725-1727.

Mansour, S.A., Mossa, A.-T.H., 2010. Oxidative damage, biochemical and histopathological alterations in rats exposed to chlorpyrifos and the antioxidant role of zinc. Pest. Biochem. Physiol. 96, 14-23.

Morton, D.B., Griffiths, P.H., 1985. Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. Vet. Rec. 116, 431-436.

NOM-062-ZOO-1999, Norma Oficial Mexicana. Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio.

Pérez-González, I.E., Prado-Ochoa, M.G., Muñoz-Guzmán, M.A., Vázquez-Valadez, V.H., Velázquez-Sánchez, A.M., Avila-Suárez, B.L., Cuenca-Verde, C., Angeles, E., Alba-Hurtado, F., 2013. Effect of new ethyl and methyl carbamates on *Rhipicephalus microplus* larvae and adult ticks resistant to conventional ixodicides. Vet. Parasitol. http://dx.doi.org/10.1016/j.vetpar.2013.07.042.

Prado-Ochoa, M.G., Gutiérrez-Amézquita, R.A., Abrego-Reyes, V.H., Velázquez-Sánchez, A.M., Muñoz-Guzmán, M.A., Ramírez-Noguera, P., Angeles, E., Alba-Hurtado, F., 2013a. Acute oral and dermal toxicity of 2 new ethyl-carbamates with activity against *Rhipicephalus microplus*.

Prado-Ochoa, M.G., Muñoz-Guzmán, M.A., Abrego-Reyes, V.H., Velázquez-Sánchez, A.M., Lara-Rocha, M., Cuenca-Verde, C., Angeles, E., Alba-Hurtado, F., 2013b. Effect of new ethyl and methyl carbamates on biological parameters and reproduction of the cattle tick *Rhipicephalus microplus*. Vet. Parasitol. 194, 49-57.

Prado-Ochoa, M.G., Ramírez-Noguera, P., Díaz-Torres, R., Garrido-Fariña, G.I., Vázquez-Valadez, V.H., Velázquez-Sánchez, A.M., Muñoz-Guzmán, M.A., Angeles, E., Alba-Hurtado, F., 2013c. The action of two ethyl carbamates on acetylcholinesterase and reproductive organs of *Rhipicephalus microplus.*

Reitman, S., Frankel, S., 1957. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am. J. Clin. Pathol. 28, 56-63.

Szasz, G., 1969. A kinetic photometric method for serum gamma-glutamyl transpeptidase. Clin. Chem. 15, 124-136.

Westgard, J.O., Poquette, M.A., 1972. Determination of serum albumin with the "SMA 12-60" by a bromcresol green dye-binding method. Clin. Chem. 18, 647-653.

Wooton, E.J., 1964. Micro-Analysis in Medical Biochemestry, fourth ed. Churchill, London, U.K.

# Capítulo 10

## GENOTOXICITY AND CYTOTOXICITY ASSESSMENT OF NEW ETHYL CARBAMATES WITH ACARICIDAL ACTIVITY

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**ABSTRACT**

Ethyl-4-bromophenyl-carbamate (LQM 919) and ethyl-4-clorophenyl-carbamate (LQM 996) are newly synthesized chemicals with acaricidal properties. Both chemicals' genotoxicity was evaluated during acute and subchronic exposures in rats. Peripheral blood smears from male Wistar rats treated once with 50 and 300 mg/kg LQM 919 or LQM 996 (acute study) and male and female Wistar rats exposed with 12.5, 25 and 50 mg/kg LQM 919 or LQM 996 for a period of 90 days (subchronic study) were analyzed for the frecuency of micronucleated normochromatic erythrocytes (MN-NCE), micronucleated polychromatic erythrocytes (MN-PCE) and the %PCE. In the acute study, both chemicals produced significant increases in MN-PCE in rats treated with 50 and 300 mg/kg. After subchronic exposure were seen significance increases in MN-PCE and MN-NCE in male and female rats exposed to 12.5, 25 and 50 mg/kg/day LQM 919 or LQM 996. Additionally we estimate the mitotic index and cell proliferation kinetics in human lymphocyte cultures. The results obtained shown that carbamate LQM 919 and LQM 996 have a significant effects on the mitotic index ($p < 0.05$) comparing with the control, and a high percentage of first division metaphases were observed in the highest dose tested (0.3mM). Therefore, the results of this study shown the cytostatic and genotoxic potencial of these chemicals in some doses tested. It is noteworthy to mention that we did not found DNA damage when the subchronic exposure was suspended, showing that the MN frequency was similar to the non-exposed animals. The results obtained in this study might suggest caution in the use of the molecules during acute and subchronic exposures when the route of administration is oral.

Keywords: carbamate, genotoxicity, citotoxicity, micronuclei, acaricide, tick, *Rhipicephalus microplus*

## INTRODUCTION

Ticks are blood-sucking arthropods that parasitize domestic animals, wildlife and humans, are the most important ectoparasites of cattle in tropical and subtropical areas of Central and South America, Africa and Australia [Baxter and Barker, 1998] *Rhipicephalus microplus* is the most important tick species in these areas because it produces large economic losses for livestock. Anemia, reduced growth, less reproductive performance, lower meat and milk production, decreased quality of fur, paralysis and transmission of diseases such as babesiosis and anaplasmosis are effects of tick infestation [Solorio-Rivera et al., 1999; Oliveira et al., 2005]. Mexico poses a serious problem for grazing livestock as it is estimated that 70% of the national livestock is exploited in regions where it occurs [Castellanos, 1998]. Acaricide resistance in the tick *R. microplus* has increased as a problem for the global livestock, and in Mexico is growing at an alarming rate because their distribution also coincides with the tropical areas, which are the most important areas of the national livestock industry [Soberanes-Céspedes et al., 2002]. In view of this problem some new carbamates were synthesized in the FES-Cuautitlán, UNAM and have been tested on the effectiveness on *R. microplus*, they were called LQM 919 and LQM 996.

These molecules were able to alter the development of *R. microplus* ticks, affecting egg laying and egg hatching in vitro after treatment of adult ticks; egg laying inhibition was observed above 50%. Morphological alterations in eggs laid by treated ticks were also observed, resulting in smaller and dry eggs, with low grip and darker than the eggs laid by the ticks from the control group; notably, the larvae hatch was inhibited in about 100% [Prado-Ochoa et al., 2013c]. In addition, carbamates have shown weak inhibition of acetylcholinesterase (AChE) activity and have a low affinity for *R. microplus* AChE. Some abnormalities and disruption of the membrane, epithelium and nuclei have shown in oocytes of carbamate-treated [Prado-Ochoa et al., 2013d]. Since the evidence has been shown that LQM 919 and

LQM 996 molecules possess properties that promise to improve the effectiveness as acaricide on *R. microplus,* the aim of this study was to evaluate the genotoxic and cytotoxic potential of these compounds. According with this, we determined two parameters associated with cytotoxicity: mitotic index and cell proliferation index in human lymphocytes to know if LQM 919 and LQM 996 carbamates alter the lymphocytes proliferation *in vitro*. We analyzed the carbamates capacity to induced micronuclei (MN) *in vivo* under acute and subchronic exposures; this assay is well-established method for evaluating the potential genotoxic effects [Hayashi et al., 1994; Witt et al., 2000]. Our data indicates that LQM 919 and LQM 996 carbamates have genotoxic and cytotoxic potential, however, when the exposure was suspended, we found that after 21 days, the MN frequency was similar to the non-exposed animals.

## MATERIALS AND METHODS

### Carbamates

The carbamates used in this study were designed and synthesized at the Laboratorio de Química Medicinal, FES-Cuautitlán, using a benzimidazole molecule as the structural base. The carbamates were synthesized by reacting aryl- and alkylamines with sodium hydride and benzene diethylcarbonate, followed by column chromatography purification; next, the products were recrystallized. The carbamates were structurally characterized through interpretations of their spectra, hydrogen and carbon-13 nuclear magnetic resonance and mass spectrometry [Angeles et al., 2000]. The chemical structures, nomenclature, molecular weights and identification codes of the carbamates are shown in Table 1.

**Mitotic and cell proliferation index in lymphocyte cultures**

Peripheral blood samples from two healthy and non smoker men (aged 22 and 32 years) were used; 0.5 mL of blood was cultured in RPMI-1640 medium (5 mL, Sigma, St. Louis, MO, USA). Lymphocyte division was stimulated by phytohemagglutinin (PHA; 0.2 mL, Gibco). Bromodeoxyuridine (SIGMA) was added at the beginning of culture in a final concentration of 32 µM. Cultures were incubated at 37°C for 72 hours, the last 24 hours the cultures were treated with LQM 919 or LQM 996 dissolved in dimethylsulfoxide (DMSO). We tested carbamate concentrations similar to those effective against ticks [Prado-Ochoa et al., 2013c] and logarithmic dilutions of these concentrations. Negative control and control with DMSO were included. Cultures were performed in duplicate and repeated at least three times. A solution of 0.2 mL of 0.04% colchicine (Research Organics) was added 2 hours before harvested. The cells were collected by centrifugation (1500 rpm/10min), the supernatant was extracted and 6 mL of KCl (0.075M) was added and allowed to stand for 30 min. Cells were fixed with a methanol-acetic acid solution (3:1) and dropped onto glass slides. Slides were stained according to the fluorescence plus Giemsa technique [Perry and Wolff, 1974] to differentiate sister chromatids.

The mitotic index (MI) was scored as the number of metaphase cells in 2000 limphocytes. The lymphocyte proliferation kinetics (LPK) was evaluated in the first 100 metaphases from each duplicate culture, determining the proportions of first (M1), second (M2), and third or more (M3) mitotic divisions according to the sister chromatids color pattern. Replication index (RI) was calculated according to the formula RI = (M1 + 2M2 + 3M3)/100 [Ivett and Tice, 1982]. The percentage of inhibition of mitotic index (% IMI) was calculated according to the formula % IMI= [1-(MIob/MIctr)] x 100, where MIob= Mitotic index of treated culture and MIctr = Mitotic index of control-DMSO culture [Elizondo et al., 1994].

**Animals Husbandry**

Clinically healthy 7-8 week old male and female Wistar rats weighing between 175 and 200 g were used. All animals were kept in groups of 3 to 5 individuals. Environmental temperature was maintained at 22 ± 2°C with a relative humidity between 30-70% and 12 x 12 light-darkness hours. They were fed with commercial feed and water *ad libitum*. This study was approved by the Internal Committee for the Care of Experimental Animals of the Postgraduate Program of Animal Production and Health (UNAM, Mexico).

**Experimental design for acute exposure**

A total of 40 male Wistar rats distributed in 9 groups of 5 rats each were used. Rats in groups 1, 2 and 3 received 50, 300 and 2000 mg/kg respectively of LQM 919 dissolved in DMSO and corn oil. Rats in groups 4, 5 and 6 received 50, 300 and 2000 mg/kg respectively of LQM 996 dissolved in DMSO and corn oil. Rats in group 6 received DMSO dissolved in corn oil, while rats in group 8 only received corn oil (control groups). Treatments were administered in a single dose using an intragastric tube. The positive control received single intraperitoneal injection of 20 mg/kg of cyclophosphamide in 0.9% saline [Krishna et al., 2000]. Two peripheral blood samples from the caudal vein were taken; the first was obtained before the carbamate exposure and the second was taken 48 hours after carbamate exposure.

**Experimental design for subchronic exposure**

A total of 82 male and female Wistar rats distributed in 9 groups were used (Table 2). The doses were selected based on our previous studies of acute oral toxicity (unpublished data). Rats were daily exposed to carbamates LQM 919 or LQM 996 in drinking water for 90 days. Carbamate water concentrations were adjusted every 7 days according to animal weight and water consumption to maintain constant the dose exposure. After 90 days of exposure, peripheral blood

samples from the caudal vein were obtained. An additional group named satellite was included; blood samples from satellite group were obtained 21 days after the carbamates exposure (25 mg/kg/day) was suspended.

**Micronucleus assay**

The micronucleus assay was performed as previously described [Salazar et al., 2013]. Briefly, peripheral blood obtained from the caudal vein was collected into vials containing heparin. Peripheral blood smears were air dried and fixed in absolute methanol. Slides were stained with acridine orange (Sigma–Aldrich) and coded before blind scoring. The frequency of micronuclei was evaluated in mature erythrocytes (normochromatic) and polychromatic erythrocytes. For each animal, 2000 uniformly stained normochromatic (NCE) and polychromatic erythrocytes (PCE) were scored per slide using a fluorescence microscope (450–490 nm excitation and 520 nm emission) and micronucleated cells (MN-NCE and MN-PCE) were counted. In addition, 2000 total erythrocytes (polychromatic and mature erythrocytes) were scored on peripheral blood slides to determine the percentage of PCE as a measure of treatment-induced bone marrow toxicity. PCE and NCE are easily differentiated by the presence or absence of residual RNA, respectively, using acridine orange. Only those cells with clearly identifiable micronuclei were considered for the analysis.

**Statistical analysis**

All data were reported as the mean ± standard deviation of the mean. The data of MN-NCE, MN-PCE, % PCE, % IMI, IR, % M1, % M2 and % M3 were analyzed by one-way ANOVA and Tukey's test for comparisons between the means with a 95% confidence limit.

**RESULTS**

In order to estimate the cytotoxic and cytostatic effects, mitotic index and cell proliferation were evaluated in lymphocytes treated with different concentrations of carbamates. The results obtained shown that both carbamates, LQM 919 and LQM 996 have a significant effects on the mitotic index ($p < 0.05$) comparing with the control (Figure 2). There was a tendency to more mitotic suppression with the LQM 996 compound. When we analyzed the differences between the concentrations of carbamates tested, the effects were no significant, although there was a significant inhibition with 0.3 mM of both carbamates ($p < 0.05$). Notably, no effects were observed on the replication rate in any of the concentrations tested, however, a high percentage of first division metaphases were observed with the high dose tested (0.3mM) data not showed. These results suggest that both carbamates have a cytostatic effect in the high concentration tested.

During the analysis we observed evidences related to genotoxic effects in lymphocytes treated with carbamates. Polyploid cells were observed in cultures exposed to LQM996 with respect to control lymphocytes in the dose 0.15 mM, suggesting that the disruption of cell spindle machinery could be involved. These effects must be studied in a future using molecular tools to be conclusive.

The frequency of micronucleus in PCE increased significantly ($p < 0.05$) in animals treated with LQM 919 (males exposed to 50 mg/kg/day) and in animals treated with LQM 996 (females and males exposed to 25 mg/kg/day In the satellite groups, no difference with respect to witness was observed. No difference was observed in the MN-PCE frequency between males and females of the same groups (Figure 3).

The effects of subchronic exposure to carbamates LQM 919 and LQM 996 on the frequency of micronuclei in erythrocytes from peripheral blood of male and female rats are shown in figure 4. The results shown a significant increment in a

manner dose response of MN-NCE frequencies observed in male and female rats when they were subchronically exposed to LQM 996 and LQM 919 carbamates comparing with the control and satellite group (21 days after the administration suspended 25 mg/kg/day). In male and female rats exposed to LQM 919 significant induction of MN-NCE did not dose response related (figure 4A) In female rats groups exposed to LQM 996 a clear dose-effect response were observed (figure 4B).. Regarding satellite groups, no significant differences were observed compared to controls of females and males in LQM 919 and LQM 996 exposures (figure 4 A and B).

By comparing the MN-PCE test results of subchronic treated rats and the acute exposure (Figures 3 and 5) acute testing according to OECD guidelines (2001) done in which logarithmic higher doses were used, the results shown that only in the highest doses tested of LQM 919 and LQM 996 (300 mg/kg), induced significant increment in MN-PCE frequency respect with the negative control (carbamates non exposed rats). When these animals were exposed to 50 mg/kg with the same carbamates no effect was observed in MN-PCE frequencies but in the subchronic exposure a dose-response effect was observed in all range doses tested.

The % PCE in the peripheral blood indicate significant changes in erythropoiesis rate in rats exposed to LQM919 and LQM 996 compared with the control in both gender (Figure 6). There was a significant increase ($p < 0.05$) compared to the control groups in the % EPC in males and females in the groups exposed to 12.5, 25 and 50 mg / kg LQM 919, in males and females exposed to 25 and 50 mg/kg/day of LQM 996 exposed females and 12.5 mg/kg/day of LQM 996. In satellite groups (LQM 919 as LQM 996), 21 days after carbamates administration were suspended (21 days after exposure), the % PCE was no different than the control groups.

## DISCUSSION

Acaricide resistance has increased and this is a problem for the global livestock. Organophosphates, pyrethroids and amidines resistance has been reported [George et al., 2004; Rodriguez-Vivas et al., 2007; Perez-Cogollo et al., 2010]. Considering the severe effects on the cattle and the resistant of *R. microplus* to conventional acaricides, new carbamates (LQM 919 and LQM 996) have been synthesized in the UNAM-FES-Cuautitlán and testing their effectiveness on the tick through disruption on *R. microplus* development [Prado-Ochoa, 2009]. From this point of view, the study of cytotoxic and genotoxic capacity associated with carbamates LQM 919 and LQM 996 in this work is of interest given the broad biological activity against ticks. Our previous results showed that these carbamates inhibit reproduction *in vitro* in tick strains of *R. microplus* and that acute and subchronic exposure to carbamates in rats is well tolerated and neither deaths or clinical signs of toxicity related to exposure were observed [Prado-Ochoa et al., 2013 a,b], showing the harmless nature of LQM 919 and LQM 996 carbamates evaluated over short and prolonged exposure,

In the current study we found that only the high concentration of both carbamates modifies the pattern of cell proliferation in comparison with the control cultures. There was an increase in the number of first division and decreased third division metaphases proportions, indicating an increase in cell cycle length or a possible cell cycle arrest in metaphase. These results are relevant considering that in previous studies of our group, the direct exposure of the tick to the LQM carbamates (*in vitro* tests) showed significant changes in the ovarian cells and eggs of the ticks, compromising their development cycle. In fact if we considered the natural process of infestation of cattle and the potential use of these molecules in tick control, it will be interesting design in vitro and in vivo toxicological profiles of these carbamates among them the toxicokinetics and toxicodynamics *in vivo.*

The erythrocyte micronucleus test *in vivo* is used for detection of damage caused by the test substances to chromosomes or to the mitotic apparatus erythroblasts, by analyzing samples of erythrocytes either bone marrow or peripheral blood animals, usually rodents. The purpose of this test is to identify whether the evaluated substances cause cytogenetic damage related with clastogenicity or aneugenicity resulting in the formation of micronuclei containing chromosome fragments or whole chromosomes [Hayashi et al., 1994,.Witt et al., 2000).

Genotoxic effects have been reported as caused by other carbamate carbosulfan [Giri et al., 2002], aldicarb [Kevekordes et al., 1996], propoxur [Gonzalez Cid et al., 1990] and ethyl carbamate [Hubner et al., 1997]. In this work, dose response effect was observed on MN-NCE frequencies in male and female rats when they were sub chronically exposed to LQM 996 and LQM 919. Only the higher tested doses of LQM 919 and LQM 996 (300 mg/kg) induced a significant increment in MN-PCE with the acute exposure, whereas with LQM 996, a higher increase of MN-PCE was evident in exposed male rats compared with females exposed to 300 mg/kg. It has been suggested that the higher potency of the exposing agent could be reaching a quick increment compared with the control group, and also the sooner it starts to decline MN frequency. The trend of non-linear increment with LQM 919 and decline of MN-NCE frequencies after a relatively long duration as subchronic exposure could be the result of overdose and toxic effect in this test system. The % PCE in the peripheral blood might suggest significant changes in erythropoiesis rate in rats exposed to LQM 919 and LQM 996 compared with the control in both gender (figure 6). The results obtained in relation to the *in vitro* cytotoxic capacity observed in human lymphocytes exposed to carbamates of interest, does not appear to be associated with the increase in the ratio of polychromatic erythrocytes in male and female rats.

It has been suggested that in which the MN frequency is diminished depend upon the toxicological magnitude of the chemical agent, especially in clastogen type. It has been suggested that the higher potency of the exposing agent could be reaching a quick increment compared with the control group, and also the sooner it starts to decline MN frequency. The trend of non-linear increment and decline of MN frequencies after a relatively long duration as in sub chronic exposure could be the result of overdose and toxic effect in this test system.

While the evidence of this study show that the compounds LQM 919 and LQM 996 at the highest doses tested have significant ability *in vivo* to induce MN, the fact that once suspended exposure, satellite groups showed decreased frequencies MN reaching baseline levels is important.

Usually acaricides are applied by spraying or immersion baths at varying intervals generally higher than a week [George et al., 2004]. If we consider the case of the treatment scheme that is intended to follow with these carbamates to control tick infestations and they could be used in the traditional way, the risk exposure may be lower. In addition to this, the evidence of direct exposure to ticks for 30 minutes with both carbamates induce significant inhibitory effects in the ovary of parasites altering their biological cycle [Prado-Ochoa et al. , 2013 c,d].

In previous reports we did not observed significant dermal alterations or toxic effects in male rats after acute dermal exposure of LQM 919 and LQM 996 (Prado-Ochoa et al., 2013b). Indeed, Perez-Gonzalez et al., 2013 also demonstrated that the carbamates were effective on strains of *R. microplus* resistant to other acaricides.

The results obtained in this study might suggest caution in the use of the molecules during acute and subchronic exposures when the route of administration is oral. Thus, still need further research that clarify the LQM 919 and LQM 996 genotoxicity under chronic exposure conditions, because genotoxicity and carcinogenicity risks of agrochemicals could depend on the duration of their actions

on cells, In addition, it is important to continue *in vivo* studies on the effectiveness of the compounds as ixodicides in order to complement the results obtained in this work. It is necessary implement *in vivo* and *in vitro* useful studies that provides key about the toxicological profile of these new potential molecules with biological activity.

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## TABLES AND FIGURES

Table 1. Chemical structures and molecular weights of the evaluated carbamates.

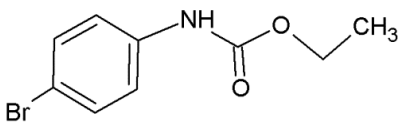
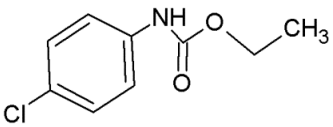
| Carbamate identification code | Chemical structure | Molecular weight (g/mol) |
|---|---|---|
| LQM 919 |  Ethyl (4-bromophenyl)carbamate | 244 |
| LQM 996 |  Ethyl (4-chlorophenyl)carbamate | 199.63 |

Table 2. Distribution of the experimental groups in subchronic study.

| Group | Treatment | Rats: number and sex | Treatment days /observation days after treatment |
|---|---|---|---|
| 1 | Control group (0.1% DMSO) | 5 males and 5 females | 90/0 |
| 2 | LQM 919 (12.5 mg/kg/day) | 5 males and 5 females | 90/0 |
| 3 | LQM 919 (25 mg/kg/day) | 5 males and 5 females | 90/0 |
| 4 | LQM 919 (50 mg/kg.day) | 5 males and 5 females | 90/0 |
| 5 | LQM 919 (25 mg/kg/day), satellite group | 3 males and 3 females | 90/21 |
| 6 | LQM 996 (12.5 mg/kg/day) | 5 males and 5 females | 90/0 |
| 7 | LQM 996 (25 mg/kg.b/day) | 5 males and 5 females | 90/0 |
| 8 | LQM 996 (50 mg/kg/day) | 5 males and 5 females | 90/0 |
| 9 | LQM 996 (25 mg/kg/day), satellite group | 3 males and 3 females | 90/21 |

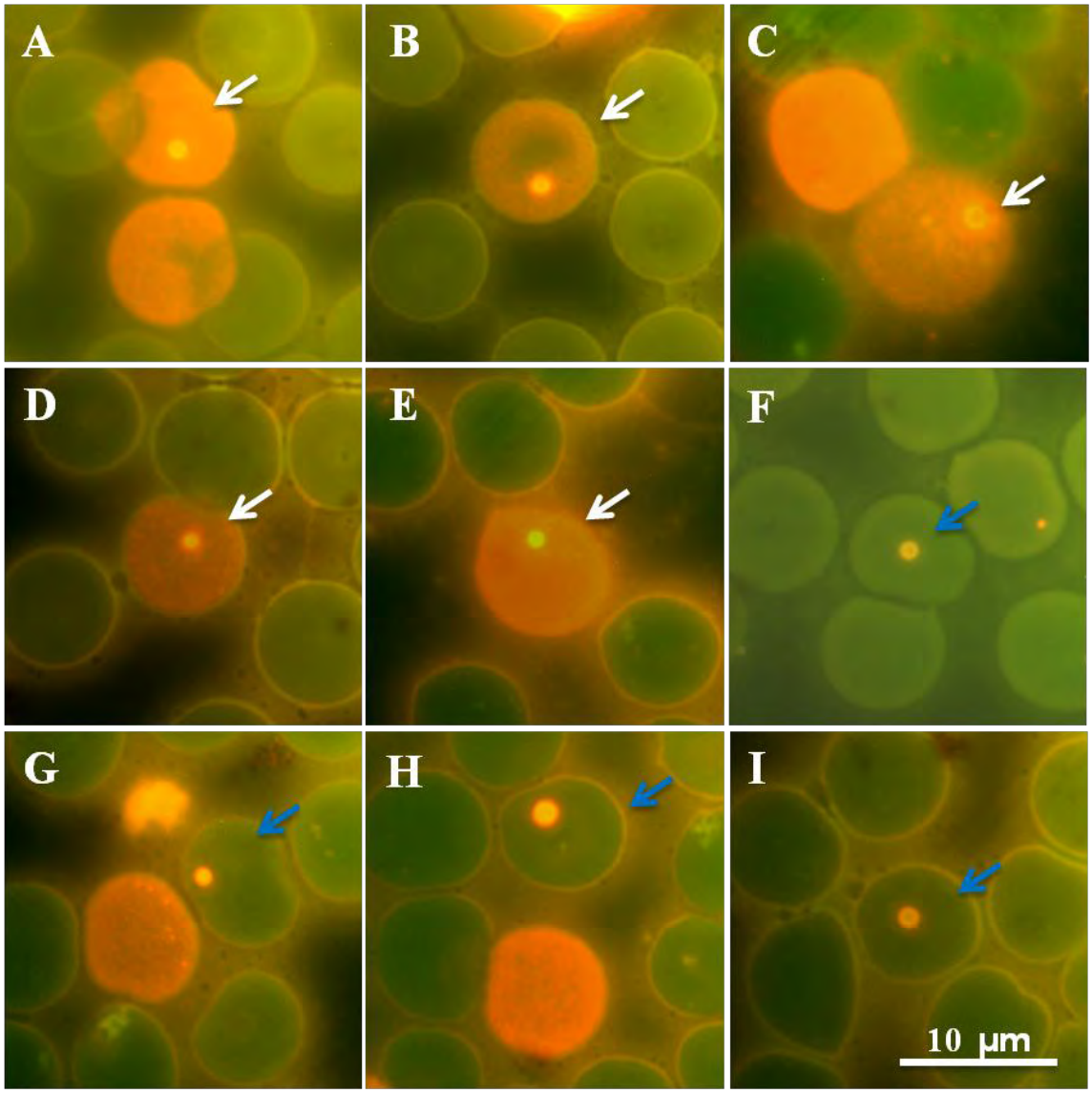



Figure 1. Micronucleated erythrocytes from Wistar rats exposed with LQM 919 or LQM 996 for a period of 90 days (subchronic study). A, F: 25 mg/kg/day LQM 919, B,I : 50 mg/kg/day LQM 919 or C,D: 25 mg/kg/day LQM 996, E,G,H: 50 mg/kg/day LQM 996. White arrows = micronucleated polychromatic erythrocytes; blue arrows = micronucleated normochromatic erythrocytes.

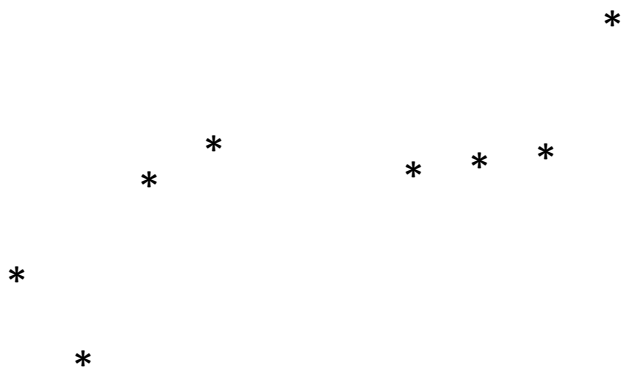


Figure 2. *In vitro* effects of different concentrations of the carbamates LQM 919 and LQM 996 on lymphocytes mitotic index as percentage of inhibition.*Statistically different from control ($p<0.05$).

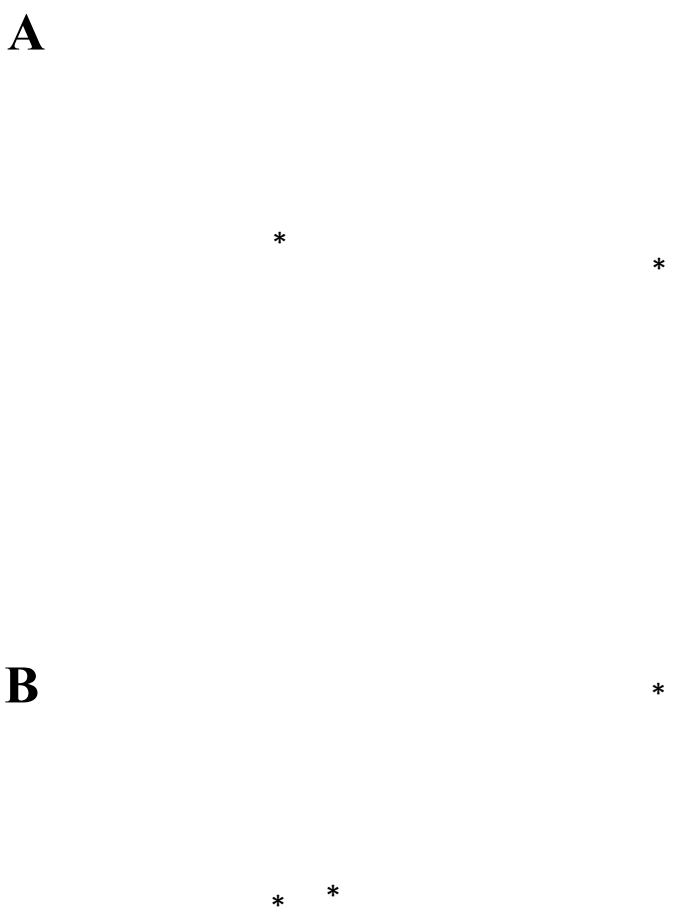



Figure 3. Micronucleated polychromatic erythrocytes induced by subchronic exposure to LQM 919 or LQM 996 in peripheral blood of females and males Wistar rats. (A) Micronucleated polychromatic erythrocytes frequency in rats sub chronically exposed to LQM 919. (B) Micronucleated polychromatic erythrocytes frequency in rats sub chronically exposed to LQM 996. st= satellite group. *Statistically different from control ($p<0.05$).

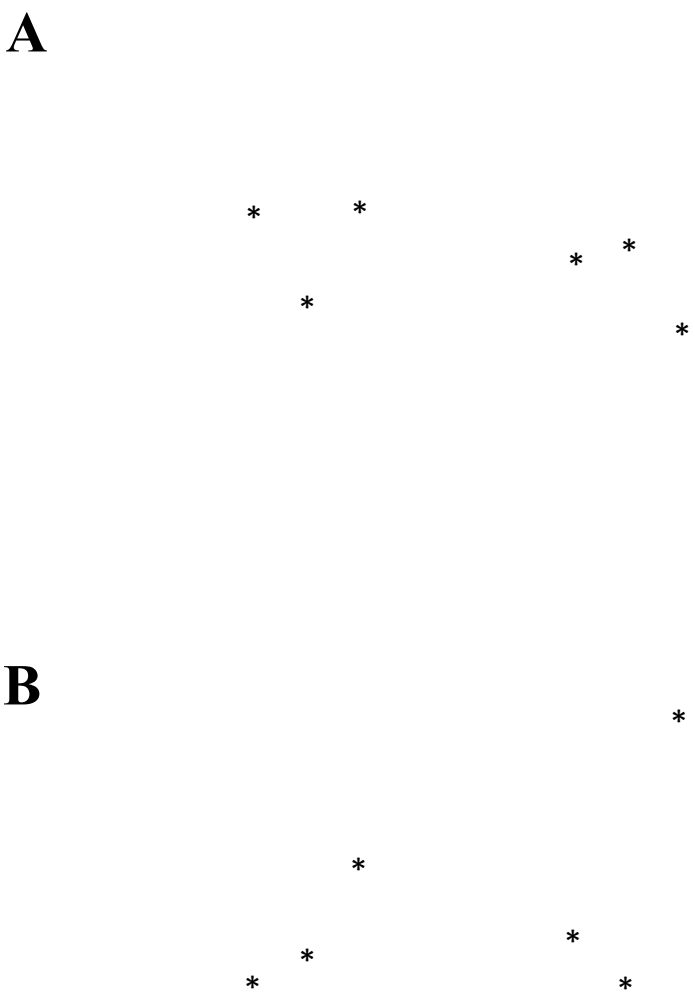



Figure 4. Micronucleated normochromatic erythrocytes induced by subchronic exposure to LQM 919 or LQM 996 in peripheral blood of females and males Wistar rats. (A) Micronucleated normochromatic erythrocytes frequency in rats sub chronically exposed to LQM 919. (B) Micronucleated normochromatic erythrocytes frequency in rats sub chronically exposed to LQM 996. st= satellite group, *Statistically different from control ($p<0.05$).

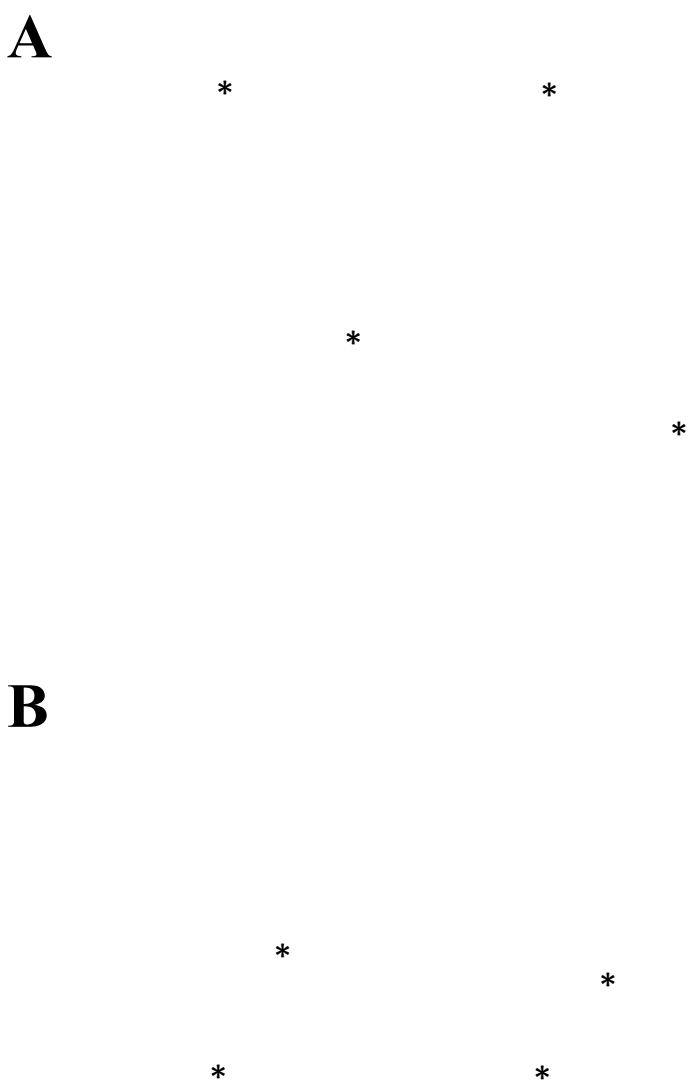



Figure 5. Micronucleated polychromatic erythrocytes induced by acute exposure to LQM 919 or LQM 996 in peripheral blood of Wistar rats. (A) Micronucleated polychromatic erythrocytes frequency in rats, 48 h after exposure to LQM 919 or LQM 996. (B) Percentage of polychromatic erythrocytes in rats, 48 h after exposure to LQM 919 or LQM 996. *Statistically different from negative control ($p<0.05$).

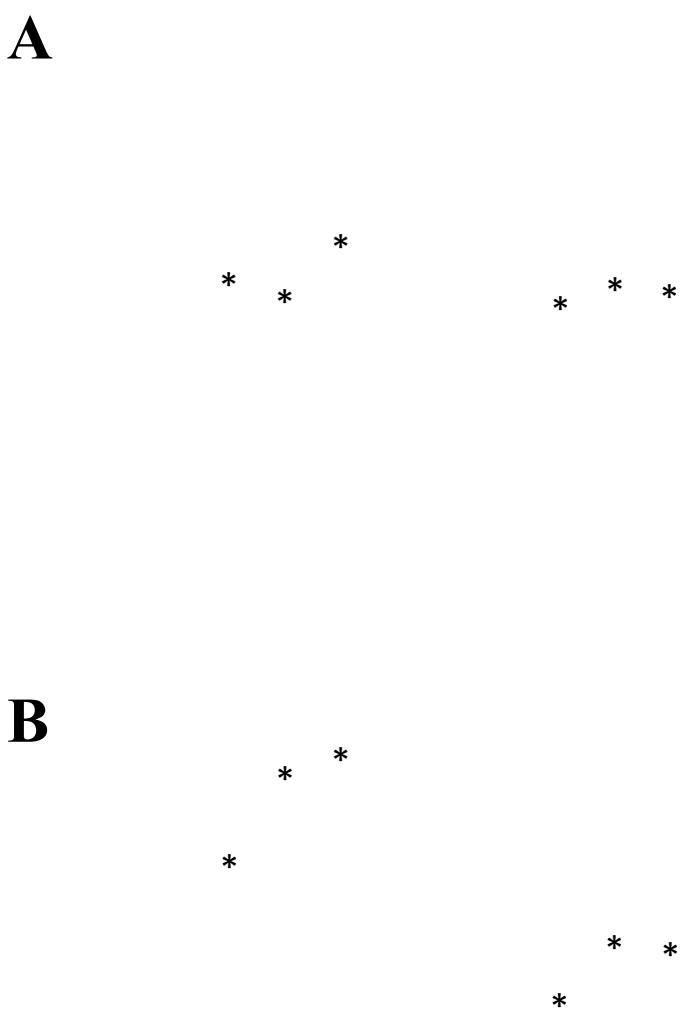



Figure 6. Percentage of polychromatic erythrocytes induced by subchronic exposure to LQM 919 or LQM 996 in peripheral blood of females and males Wistar rats. (A) Percentage of polychromatic erythrocytes in rats sub chronically exposed to LQM 919. (B) Percentage of polychromatic erythrocytes in rats sub chronically exposed to LQM 996. st= satellite group. *Statistically different from control ($p<0.05$).

## REFERENCES

Angeles E, Mart nez P, Keller J, Mart nez R, Rubio M, Ram rez G, Castillo R, López-Castañares R, Jiménez E. 2000. Ethyl and methylphenylcarbamates as antihelmintic agents: theoretical study for predicting their biological activity by PM3. Journal of Molecular Structure: THEOCHEM 504(1):141-170.

Baxter GD, Barker SC. 1998. Acetylcholinesterase cDNA of the cattle tick, Boophilus microplus: characterisation and role in organophosphate resistance. Insect Biochem Mol Biol 28(8):581-589.

Bonassi S, El-Zein R, Bolognesi C, Fenech M. 2011. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. Mutagenesis 26(1):93-100.

Castellanos, J. 1998. Seguimiento a predios con garrapatas resistentes hacia los ixodicidas y alternativas para su control. Curso de diagnóstico y control de las principales enfermedades parasitarias. Ciudad Victoria, Tamaulipas, México

Elizondo G, Montero R, Herrera JE, Hong E, Ostrosky-Wegman P. 1994. Lymphocyte proliferation kinetics and sister-chromatid exchanges in individuals treated with metronidazole. Mutat Res 305(2):133-137.

George JE, Pound JM, Davey RB. 2004. Chemical control of ticks on cattle and the resistance of these parasites to acaricides. Parasitology 129 Suppl:S353-366.

Giri S, Giri A, Sharma GD, Prasad SB. 2002. Mutagenic effects of carbosulfan, a carbamate pesticide. Mutat Res 519(1-2):75-82.

Gonzalez Cid M, Loria D, Matos E. 1990. Genotoxicity of the pesticide propoxur and its nitroso derivative, NO-propoxur, on human lymphocytes in vitro. Mutat Res 232(1):45-48.

Hayashi M, Tice RR, MacGregor JT, Anderson D, Blakey DH, Kirsh-Volders M, Oleson FB, Jr., Pacchierotti F, Romagna F, Shimada H, et al. 1994. In vivo rodent erythrocyte micronucleus assay. Mutat Res 312(3):293-304.

Heddle JA, Hite M, Kirkhart B, Mavournin K, MacGregor JT, Newell GW, Salamone MF. 1983. The induction of micronuclei as a measure of genotoxicity. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat Res 123(1):61-118.

Hubner P, Groux PM, Weibel B, Sengstag C, Horlbeck J, Leong-Morgenthaler PM, Luthy J. 1997. Genotoxicity of ethyl carbamate (urethane) in Salmonella, yeast and human lymphoblastoid cells. Mutat Res 390(1-2):11-19.

Ivett J, Tice R. 1982. Average generation time: a new method of analysis and quantitation of cellular proliferation kinetics. Environ Mutagen 4:358.

Kevekordes S, Gebel T, Pav K, Edenharder R, Dunkelberg H. 1996. Genotoxicity of selected pesticides in the mouse bone-marrow micronucleus test and in the sister-chromatid exchange test with human lymphocytes in vitro. Toxicology Letters 89(1):35-42.

Kirkland DJ, Henderson L, Marzin D, Muller L, Parry JM, Speit G, Tweats DJ, Williams GM. 2005. Testing strategies in mutagenicity and genetic toxicology: an appraisal of the guidelines of the European Scientific Committee for Cosmetics and Non-Food Products for the evaluation of hair dyes. Mutat Res 588(2):88-105.

Krishna G, Urda G, Paulissen J. 2000. Historical vehicle and positive control micronucleus data in mice and rats. Mutat Res 453(1):45-50.

Morita T, Asano N, Awogi T, Sasaki YF, Sato S, Shimada H, Sutou S, Suzuki T, Wakata A, Sofuni T, Hayashi M. 1997. Evaluation of the rodent micronucleus assay in the screening of IARC carcinogens (groups 1, 2A and 2B) the summary report of the 6th collaborative study by CSGMT/JEMS MMS. Collaborative Study of the Micronucleus Group Test. Mammalian Mutagenicity Study Group. Mutat Res 389(1):3-122.

OECD. 2001. Guideline for testing of chemicals. Acute Oral Toxicity-Fixed Dose Procedure. Guideline 420.

Oliveira MC, Oliveira-Sequeira TC, Araujo JP, Jr., Amarante AF, Oliveira HN. 2005. Babesia spp. infection in *Boophilus microplus* engorged females and eggs in Sao Paulo State, Brazil. Vet Parasitol 130(1-2):61-67.

Perez-Cogollo LC, Rodriguez-Vivas RI, Ramirez-Cruz GT, Miller RJ. 2010. First report of the cattle tick *Rhipicephalus microplus* resistant to ivermectin in Mexico. Vet Parasitol 168(1-2):165-169.

Pérez-González IE, Prado-Ochoa MG, Muñoz-Guzmán MA, Vázquez-Valadez VH, Velázquez-Sánchez AM, Avila-Suárez BL, Cuenca-Verde C, Angeles E, Alba-Hurtado F. 2013. Effect of new ethyl and methyl carbamates on

*Rhipicephalus microplus* larvae and adult ticks resistant to conventional ixodicides. Vet Parasitol http://dxdoiorg/101016/jvetpar201307042(0).

Perry P, Wolff S. 1974. New Giemsa method for the differential staining of sister chromatids. Nature 251(5471):156-158.

Prado-Ochoa MG. 2009. Evaluación in vitro de la eficacia de nuevos carbamatos sobre garrapatas *Boophilus microplus* [Tesis de Maestría]. México: UNAM.

Prado-Ochoa MG, Abrego-Reyes VH, Velázquez-Sánchez AM, Muñoz-Guzmán MA, Ramírez-Noguera P, Angeles E, Alba-Hurtado F. 2013a. Subchronic toxicity study in rats of two new ethyl-carbamates with acaricidal activity.

Prado-Ochoa MG, Gutiérrez-Amézquita RA, Abrego-Reyes VH, Velázquez-Sánchez AM, Muñoz-Guzmán MA, Ramírez-Noguera P, Angeles E, Alba-Hurtado F. 2013b. Assesment of acute oral and dermal toxicity of 2 ethyl-carbamates with activity against *Rhipicephalus microplus* in rats.

Prado-Ochoa MG, Muñoz-Guzmán MA, Abrego-Reyes VH, Velázquez-Sánchez AM, Lara-Rocha M, Cuenca-Verde C, Angeles E, Alba-Hurtado F. 2013c. Effect of new ethyl and methyl carbamates on biological parameters and reproduction of the cattle tick *Rhipicephalus microplus*. Veterinary Parasitology 194(1):49-57.

Prado-Ochoa MG, Ramírez-Noguera P, Díaz-Torres R, Garrido-Fariña GI, Vázquez-Valadez VH, Velázquez-Sánchez AM, Muñoz-Guzmán MA, Angeles E, Alba-Hurtado F. 2013d. The action of two ethyl carbamates on acetylcholinesterase and reproductive organs of *Rhipicephalus microplus***.**

Rodriguez-Vivas RI, Rivas AL, Chowell G, Fragoso SH, Rosario CR, Garcia Z, Smith SD, Williams JJ, Schwager SJ. 2007. Spatial distribution of acaricide profiles (*Boophilus microplus* strains susceptible or resistant to acaricides) in southeastern Mexico. Vet Parasitol 146(1-2):158-169.

Salazar AM, Mendlovic F, Cruz-Rivera M, Chavez-Talavera O, Sordo M, Avila G, Flisser A, Ostrosky-Wegman P. 2013. Genotoxicity induced by *Taenia solium* and its reduction by immunization with calreticulin in a hamster model of taeniosis. Environ Mol Mutagen 54(5):347-353.

Soberanes-Céspedes N, Santamaría-Vargas M, Sánchez-Fragoso H, García-Vázquez Z. 2002. Primer caso de resistencia al amitraz en la garrapata del ganado *Boophilus microplus* en México Téc Pecu Méx 40(1):81-92.

Solorio-Rivera JL, Rodriguez-Vivas RI, Perez-Gutierrez E, Wagner G. 1999. Management factors associated with *Babesia bovis* seroprevalence in cattle from eastern Yucatan, Mexico. Prev Vet Med 40(3-4):261-269.

The Collaborative Study Group for the Micronucleus T. 1992. Micronucleus test with mouse peripheral blood erythrocytes by acridine orange supravital staining: The summary report of the 5th collaborative study by CSGMT/JEMS · MMS. Mutation Research/Genetic Toxicology 278(2–3):83-98.

Witt KL, Knapton A, Wehr CM, Hook GJ, Mirsalis J, Shelby MD, MacGregor JT. 2000. Micronucleated erythrocyte frequency in peripheral blood of B6C3F(1) mice from short-term, prechronic, and chronic studies of the NTP carcinogenesis bioassay program. Environ Mol Mutagen 36(3):163-194

# Capítulo 11

## DISCUSIÓN

*R. microplus* se encuentra distribuida en las zonas tropicales y subtropicales del mundo, causando grandes pérdidas económicas a la ganadería. Los ixodicidas químicos han desempeñado un papel fundamental en el control de garrapatas, sin embargo, como consecuencia de su uso extensivo, *R. microplus* ha desarrollado resistencia a todas las clases de ixodicidas disponibles (Rodríguez Vivas et al., 2012). Una alternativa para el manejo de la resistencia es el desarrollo de nuevas moléculas para las cuales no se haya desarrollado resistencia. Por lo anterior, en la primera parte de este trabajo (Capítulo 6), se evaluó el efecto una serie de nuevos fármacos derivados del ácido carbámico, diseñados y sintetizados en FES Cuatitlán, sobre larvas y adultas de *R. microplus* de una cepa susceptible a ixodicidas convencionales (Prado-Ochoa et al., 2013), en este estudio se demostró que seis de estos carbamatos tienen actividad sobre el desarrollo de *R. microplus,* afectando los parámetros de oviposición y eclosión después del tratamiento *in vitro* de garrapatas hembras adultas repletas.

Paralelamente, en un trabajo realizado por nuestro grupo de investigación, se evaluó el efecto de estos carbamatos sobre dos cepas de *R. microplus* que han sido reportadas como resistentes a ixodicidas convencionales y una cepa de campo (Pérez-González et al., 2013; Prado-Ochoa et al., 2013). Las cepas utilizadas fueron: la cepa "San Alfonso" (resistente a organofosforados, amidinas y piretroides) y la cepa "La Mora" que es resistente a organofosforados y piretroides (Alonso-Díaz et al., 2006; Domínguez-García et al., 2010; Soberanes-Céspedes et al., 2002). Los carbamatos evaluados mostraron efectos similares en las cepas resistentes a los observados con la cepa susceptible. Los resultados de estos estudios mostraron que los carbamatos evaluados interfieren con el ciclo biológico de las diferentes cepas de *R. microplus*, sean susceptibles o resistentes a ixodicidas comerciales. Los

resultados obtenidos en este trabajo, sugieren que los nuevos carbamatos podrían ser una buena opción terapeútica para el control de las garrapatas *R. microplus*, es importante dilucidar su mecanismo de acción y realizar estudios de toxicidad para que en futuros estudios sea evaluada su eficacia sobre *R. microplus* en pruebas *in vivo* y de campo.

Los trabajos anteriores, junto con la evaluación del efecto de estos carbamatos sobre *Haemonchus contortus*, *Hymenolepis nana y Entamoeba histolytica*, mostraron el efecto de estos carbamatos sobre parásitos, lo que permitió registrar la patente titulada "Uso de derivados del ácido carbámico como antiparasitarios" con registro ante el Instituto Mexicano de la Propiedad Industrial NO. MX/E/2011/061614 (Anexo 1).

Las moléculas evaluadas en estos trabajos pertenecen al grupo químico de los carbamatos, algunos carbamatos como el propoxur y el carbaril tienen como mecanismo de acción la inhibición de la enzima AChE en el sistema nervioso de los artrópodos, lo que les produce parálisis y muerte (Mutero et al., 1994). Se ha reportado que existe resistencia cruzada entre organofosforados y algunos carbamatos que comparten el mismo mecanismo de acción. Los resultados que hemos obtenido en nuestro laboratorio (Pérez-González et al., 2013; Prado-Ochoa et al., 2013) mostraron que el efecto de los nuevos carbamatos era sobre la reproducción de la garrapata y que no había resistencia cruzada con los organofosforados, lo que nos hizo pensar que su mecanismo de acción es diferente al reportado para otros carbamatos usados como plaguicidas, por lo que se planteo un segundo trabajo para tratar de contestar esta pregunta. En éste se evaluó el mecanismo de acción de los dos carbamatos que según los trabajos anteriores habían tenido mayor efecto (LQM 919 y LQM 996) sobre hembras repletas de *R. microplus* (Capítulo 7). Los resultados de este estudio mostraron que los carbamatos evaluados son inhibidores débiles y con poca afinidad a la AChE de *R. microplus*. Al mismo tiempo observamos que las hembras repletas tratadas con los carbamatos evaluados

presentaron alteraciones morfológicas en los órganos reproductivos, lo que sugiere que los carbamatos evaluados tienen una acción directa sobre la vitelogénesis y la viabilidad de las células del ovario, lo que repercute en la morfología y viabilidad de los huevos producidos. Estos resultados mostraron que a diferencia de otros carbamatos, la inhibición de la AChE no es su principal mecanismo de acción, sino que éste es la inhibición de la ovogénesis.

Los resultados de los primeros estudios mostraron la eficacia de estas moléculas sobre *R. microplus* y su potencial para el control de garrapatas, sin embargo, antes de considerarse la utilización de estos nuevos carbamatos para el control de garrapatas, es necesario evaluar los efectos adversos que pudieran provocar en mamíferos. Por lo anterior, en el siguiente trabajo de esta tesis se determinó la toxicidad aguda de los carbamatos evaluados (Capítulo 8). La evaluación de la toxicidad aguda se realizó con pruebas recomendadas por la Organización para la Cooperación y Desarrollo Económico de los Estados (OECD, 2001) usando la vía probable de exposición de acuerdo a los resultados de estudios previos, a fin de proporcionar información sobre los riesgos a la salud que puedan derivarse de la exposición a corto plazo. La vía propuesta de administración de los nuevos carbamatos en bovinos, es la vía dérmica, por medio de baños de aspersión o inmersión, por otro lado, la vía dérmica también constituye la de mayor riesgo de contacto accidental del humano con los productos ixodicidas. Además, debido al hábito de acicalamiento que presentan los bovinos, estos pueden ingerir los productos utilizados en los baños, por lo que se requiere evaluar su toxicidad oral y dérmica aguda.

En el capítulo 8 se muestra que los carbamatos evaluados son de baja toxicidad oral aguda en ratas y de acuerdo a su $DL_{50}$ (300-2000 mg/kg) se clasificaron dentro de la categoría 4 (baja peligrosidad) según la Globally Harmonized System (GHS). La toxicidad dérmica aguda fue baja ($DL_{50}$>5000 mg/kg) y según los criterios de GHS, estos carbamatos se clasifican dentro de la

categoría 5 (baja toxicidad aguda). Altas dosis orales de los carbamatos produjeron signos clínicos en las ratas y un leve daño hepático y renal, identificándose estos órganos, como órganos diana de la toxicidad de estos carbamatos. La exposición dérmica no provoco eritema, edema o corrosión en la piel de las ratas expuesta a los carbamatos. En este trabajo, aunque no se midieron los niveles plasmáticos de los carbamatos evaluados, se observaron mayor cantidad de efectos tóxicos (signos clínicos, lesiones y alteraciones funcionales) en las ratas tratadas por vía oral que en las ratas tratadas por vía dérmica, lo que sugiere que estos compuestos se absorben bien en el tracto digestivo y de forma limitada por la piel. Considerando lo anterior, es importante evaluar en futuros estudios la absorción, distribución, metabolismo y excreción de estos carbamatos.

Los estudios de toxicidad oral subcrónica evalúan los efectos adversos por la exposición prolongada de una sustancia en los animales, y proveen información acerca de sus efectos negativos en la salud de los mismos, debidos a la exposición repetida, incluyendo toxicidad de órganos diana y efectos acumulativos. Además, estos estudios determinan la dosis a la cual no se presentan efectos adversos observables. Por lo anterior se determinó la toxicidad oral subcrónica (90 días) de los carbamatos LQM 919 y LQM 996 en ratas Wistar hembras y machos (Capítulo 9). La exposición a los carbamatos evaluados fue bien tolerada y la ausencia de muertes o signos clínicos de toxicidad relacionados a la exposición son un indicador de la baja toxicidad de la exposición prolongada a los carbamatos evaluados. Sin embargo, la exposición a las dosis mayores de los carbamatos evaluados indujo la elevación de las enzimas AST y GGT en plasma, lo cual junto con las alteraciones histopatológicas leves observadas en hígado y el aumento moderado de las especies reactivas al ácido tiobarbitúrico (daño oxidativo) muestran que estos carbamatos son ligeramente hepatotóxicos. De acuerdo a los resultados de este estudio se estableció que la dosis sin efectos adversos observables (NOAEL) para ambos carbamatos fue de 12.5 mg/kg/día en ratas de ambos sexos.

Por otra parte, se han reportado efectos genotóxicos ocasionados por carbamatos como carbosulfán, aldicarb (Kevekordes et al., 1996), propoxur (Gonzalez Cid et al., 1990) y etil carbamatos (Hubner et al., 1997), por lo que consideramos de gran importancia determinar si estos nuevos carbamatos producen genotoxicidad. Por lo anterior en esta tesis se fue evaluó si los carbamatos LQM 919 y LQM 996 producen daño genotóxico en ratas expuestas a estos carbamatos de forma aguda y subcrónica (Capítulo 10). En este estudio, se mostró que estos carbamatos inducen un aumento en el porcentaje de reticulocitos micronucleados en sangre periférica de ratas expuestas a 50 y 300 mg/kg de LQM 919 y LQM 996 en forma aguda, y en ratas expuestas a 25 y 50 mg/kg/día por 90 días. El estudio subcrónico mostró que estos efectos fueron reversibles después de suspender la exposición a los carbamatos. Los resultados de este estudio mostraron el potencial genotóxico de los carbamatos estudiados en algunas de las dosis empleadas, cuando son administrados por vía oral, tambien se mostró que al suspender la exposición a los carbamatos, estos efectos son reversibles.

**Perspectivas**

En la presente tesis se realizaron avances significativos en el conocimiento del efecto de los nuevos carbamatos sobre *R. microplus*, se mostró la eficacia de éstos sobre los parámetros reproductivos y desarrollo de los ovocitos de *R. microplus* y se hizo un primer acercamiento al conocimiento de su mecanismo de acción. Futuros estudios permitirán conocer los eventos celulares y moleculares asociados a su mecanismo de acción.

Así mismo, se realizaron avances en el conocimiento del potencial tóxico de estos carbamatos en mamíferos. Su baja toxicidad aguda, hace factible su utilización en pruebas sobre bovinos en las que se evalúe la eficacia de estos carbamatos sobre las diferentes fases de la garrapata. Por lo que en una siguiente etapa de esta investigación sugerimos realizar pruebas de establo y pruebas de campo. Con esto

podremos conocer si en respuesta a la exposición existe una susceptibilidad individual al efecto de los carbamatos *in vivo*, la toxicocinética que presentan entre muchos otros eventos asociados.

Los estudios de toxicidad aguda y subcrónica de estos nuevos carbamatos permitieron conocer los riesgos de exposición a altas dosis de los carbamatos por vía oral, así como de la exposición repetida por vía oral, y se estableció la dosis a la cual no se observaron efectos adversos (NOAEL), lo que permite establecer un margen de seguridad en la utilización de estos carbamatos. Con base en los resultados obtenidos, sugerimos continuar estudiando los mecanismos de toxicidad involucrados en la exposición a los carbamatos LQM 919 y LQM996. Los efectos asociados a la modulación del estrés oxidativo celular y otras señales celulares involucradas en su efecto terapeútico asociado así como en el mecanismo de acción que exhiben. Será importante desarrollar estudios multidisciplinarios e interdisciplinarios con objeto de establecer el riesgo de utilización de estos carbamatos en la salud como toxicidad crónica, teratogenicidad y carcinogenicidad, así como la evaluación de la toxicidad ambiental. Además, son necesarios estudios de absorción, distribución, metabolismo y excreción de estas nuevas moléculas.

En conclusión, la eficacia de los nuevos carbamatos sobre *R. microplus*, tanto en cepas susceptibles como resistentes a ixodicidas comerciales, el mecanismo de acción diferente a los ixodicidas comerciales y su baja toxicidad en mamíferos, hace factible el desarrollo de nuevas formulaciones farmacéuticas con estas nuevas moléculas para el control de garrapatas. Sin embargo, de acuerdo a los efectos tóxicos observados, se sugiere precaución y continuar con el estudio de los riesgos a la salud.

# Capítulo 12

## CONCLUSIONES

- Los nuevos carbamatos diseñados y sintetizados en FES-Cuautitlán son efectivos sobre garrapatas *R. microplus*, afectando sus parámetros reproductivos, ya que disminuyen la oviposición e inhiben la eclosión de larvas.

- Los carbamatos LQM 919 y LQM 996 son inhibidores débiles y con poca afinidad a la AChE de *R. microplus*.

- Los carbamatos evaluados principalmente actúan sobre las células del ovario de las garrapatas inhibiendo la vitelogénesis y el desarrollo de los ovocitos, siendo estos efectos independientes de la inhibición de la acetilcolinesterasa.

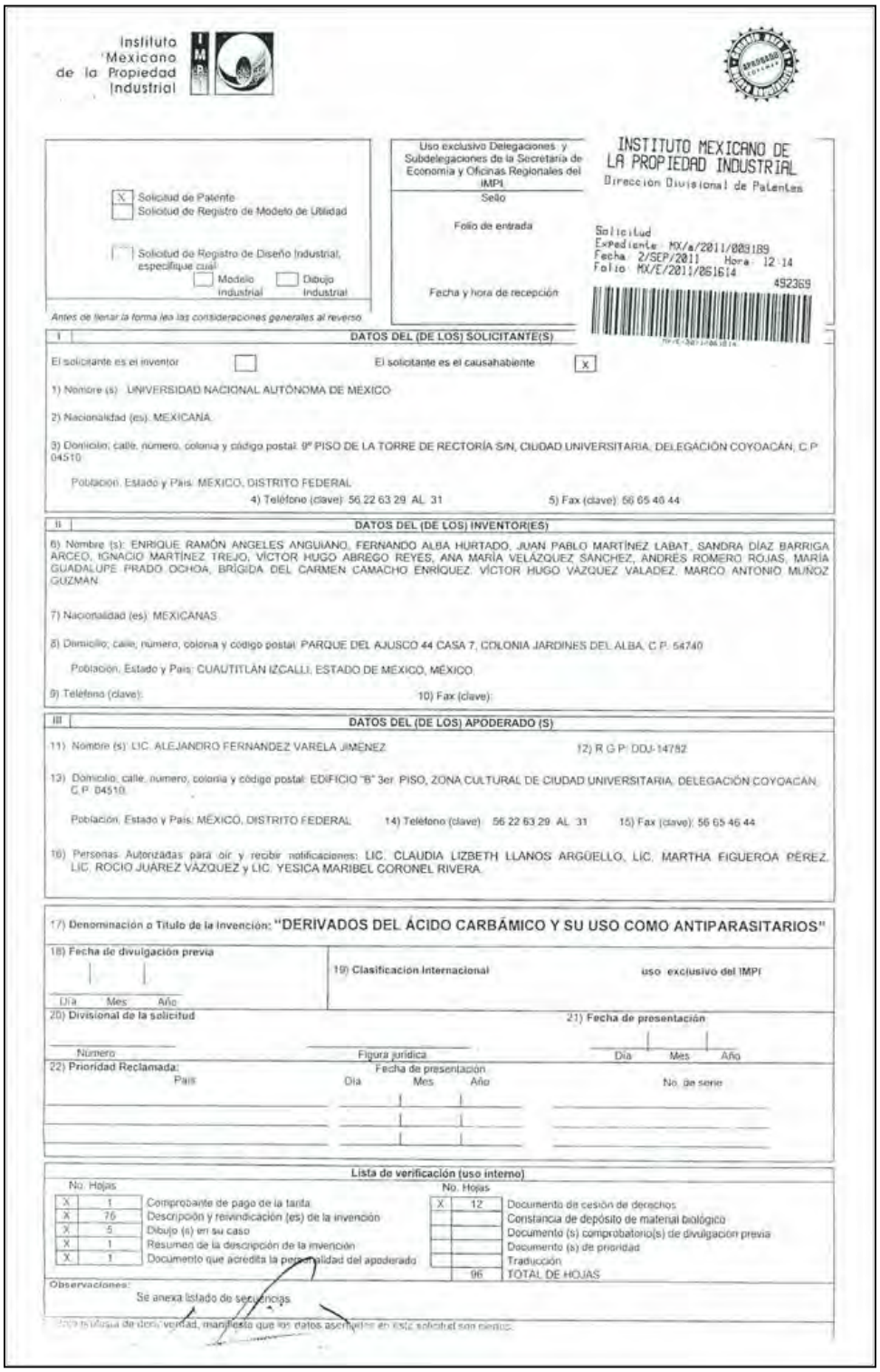
- De acuerdo a los estudios de toxicidad aguda, los nuevos carbamatos se clasifican como de baja peligrosidad por la vía oral ($DL_{50}$ en ratas de 300-2000 mg/kg) y de baja toxicidad por vía dérmica ($DL_{50}$ en ratas >5000 mg/kg) y se identificaron como órganos blanco de la toxicidad de estos carbamatos, el hígado y el riñón.

- El estudio toxicidad subcrónica indicó que estos carbamatos son de baja toxicidad cuando son administrados por un periodo prolongado y se estableció como NOAEL (nivel sin efectos adversos observables) la dosis de 12.5 mg/kg/día.

- En los estudios de genotoxicidad *in vivo*, se presentó un aumento de reticulocitos con micronúcleos a partir de la dosis de 25 mg/kg/día, lo que mostró el potencial genotóxico de estos carbamatos al ser administrados por vía oral de forma aguda o subcrónica.

# Anexo 1:

**Registro Patente No. MX/E/2011/061614**



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[X] Solicitud de Patente
[ ] Solicitud de Registro de Modelo de Utilidad
[ ] Solicitud de Registro de Diseño Industrial, especifique cual: [ ] Modelo Industrial [ ] Dibujo Industrial

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17) Denominación o Título de la invención: "DERIVADOS DEL ÁCIDO CARBÁMICO Y SU USO COMO ANTIPARASITARIOS"

18) Fecha de divulgación previa (Día Mes Año)

19) Clasificación Internacional uso exclusivo del IMPI

20) Divisional de la solicitud (Número, Figura jurídica)

21) Fecha de presentación (Día Mes Año)

22) Prioridad Reclamada: País, Fecha de presentación (Día Mes Año), No. de serie

Lista de verificación (uso interno)

| No. Hojas | | | No. Hojas | |
|---|---|---|---|---|
| X | 1 | Comprobante de pago de la tarifa | X 12 | Documento de cesión de derechos |
| X | 76 | Descripción y reivindicación (es) de la invención | | Constancia de depósito de material biológico |
| X | 5 | Dibujo (s) en su caso | | Documento (s) comprobatorio(s) de divulgación previa |
| X | 1 | Resumen de la descripción de la invención | | Documento (s) de prioridad |
| X | 1 | Documento que acredita la personalidad del apoderado | | Traducción |
| | | | 96 | TOTAL DE HOJAS |

Observaciones:
Se anexa listado de secuencias

Bajo protesta de decir verdad, manifiesto que los datos asentados en esta solicitud son ciertos.

# Referencias

Aguirre, J., Santamaría, V., 1986. Purificación y caracterización toxicológica de garrapatas *Boophilus microplus* resistentes a ixodicidas organofosforados y organoclorados. VII Reunión Anual Asc. Mex. de Parasitología Veterinaria, A.C. Ciudad Victoria, Tamps. México.

Alcántara, A.C.A., Morales, M.G.A., 2005. Evaluación del daño al DNA, producido por un grupo de derivados del ácido carbámico con acción antiparasitaria, mediante el ensayo cometa. Tesis de Licenciatura. UNAM, México.

Alonso-Díaz, M., Rodríguez-Vivas, R., Fragoso-Sánchez, H., 2006. Resistencia de la garrapata *Boophilus microplus* a los ixodicidas. Arch. med. Vet. 38, 105-113.

Anderson, J.F., Magnarelli, L.A., 2008. Biology of ticks. Infect. Dis. Clin. North Am. 22, 195-215, v.

Angeles, E., Mart nez, P., Keller, J., Mart nez, R., Rubio, M., Ram rez, G., Castillo, R., López-Castañares, R., Jiménez, E., 2000. Ethyl and methylphenylcarbamates as antihelmintic agents: theoretical study for predicting their biological activity by PM3. J. Mol. Struct.: THEOCHEM 504, 141-170.

Atkinson, P. W., Binnington K. C., Roulston, W. J., 1974. High monoamine oxidase activity in the tick *Boophilus microplus,* and inhibition by chlordimeform and related pesticides. J. Aust. Entomol. Soc.13: 207-210.

Baffi, M.A., Pereira, C.D., Souza, G.R.L.d., Ceron, C.R., Bonetti, A.M., 2007. Esterase profile in the postembryonic development of *Rhipicephalus microplus*. Pes. Agrop. Bras. 42, 1183-1188.

Barker, S.C., Murrell, A., 2004. Systematics and evolution of ticks with a list of valid genus and species names. Parasitology 129 Suppl, S15-36.

Baron, R.L., Hayes, W.J., Jr., E.R., Jr., L., 1991. Carbamate insecticides. In Handbook of Pesticide Toxicology. Academic Press, New York, NY. 3-6.

Baxter, G.D., Barker, S.C., 1998. Acetylcholinesterase cDNA of the cattle tick, Boophilus microplus: characterisation and role in organophosphate resistance. Insect Biochem. Mol. Biol. 28, 581-589.

Bernabe-Pérez, A.G., 2007. Evaluación antiparasitaria de nueve principios de síntesis derivados del ácido carbámico utilizando como modelo el cestodo *Hymenolepis nana var. fraterna* en ratones de la cepa CD1 con infestación inducida. Tesis de Licenciatura. UNAM, México.

Bernal, S., 2000. Evaluación de algunos productos derivados del ácido carbámico como agentes antimicrobianos. Tesis de Licenciatura. UNAM, México.

Botana López, L.M., Landoni, M.F., Martín-Jiménez, T., 2002. Farmacología y terapéutica veterinaria. Mc Graw Hill. Interamericana.Brkic, D.V., Vitorovic, S., Gasic, S.M., Neskovic, N.K., 2008, Carbofuran in water: Subchronic toxicity to rats. Environ. Toxicol. Pharmacol. 25, 334-341.

Carvalho, K.P., Gadelha, A.P.R., 2007. Effects of three benzimidazoles on growth, general morphology and ultrastructure of *Tritrichomonas foetus*. FEMS microbiol. Let. 275, 292-300.

Córdoba, D., 2006. Toxicología (5a ed.). Bogotá, Colombia: Ed. Manual Moderno.

Chauhan, L.K., Pant, N., Gupta, S.K., Srivastava, S.P., 2000. Induction of chromosome aberrations, micronucleus formation and sperm abnormalities in mouse following carbofuran exposure. Mutat Res 465, 123-129.

Chávez, B., Cedillo-Rivera, R., Martínez-Palomo, A., 1992, *Giardia lamblia*: ultrastructural study of the in vitro effect of benzimidazoles. J. Eukaryotic Microbiol. 39, 510-515.

Chen, A.C., He, H., Davey, R.B., 2007. Mutations in a putative octopamine receptor gene in amitraz-resistant cattle ticks. Vet. Parasitol. 148, 379-383.

Domínguez-García, D.I., Rosario-Cruz, R., Almazán-García, C., Oaxaca, J.A.S., De la Fuente, J., 2010. Resistencia a los acaricidas y su impacto en la salud animal. Trop. Subtrop. Agroecosys. 12, 181-192.

Dominguez-García, D.I., Rosario-Cruz, R., García, C.A., Oaxaca, J.A.S., De la Fuente, J., 2009. *Boophilus microplus*: biological and molecular aspects of acaricide resistance and their impact on animal health. Trop. Subtrop. Agroecosyst. 12, 181-192.

Estrela, A., Seixas, A., Termignoni, C., 2007. A cysteine endopeptidase from tick (*Rhipicephalus (Boophilus) microplus*) larvae with vitellin digestion activity. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 148, 410-416.

Fernández Ruvalcaba, M., Zhioua, E., García Vázquez, Z., 2012. Infectividad de *Metarhizium anisopliae* en contra de cepas de garrapata *Boophilus microplus* sensible y resistente a los organofosforados. Revista Mexicana de Ciencias Pecuarias 43, 433 a 440.

Foil, L.D., Coleman, P., Eisler, M., Fragoso-Sanchez, H., Garcia-Vazquez, Z., Guerrero, F.D., Jonsson, N.N., Langstaff, I.G., Li, A.Y., Machila, N., Miller, R.J., Morton, J., Pruett, J.H., Torr, S., 2004. Factors that influence the prevalence of acaricide resistance and tick-borne diseases. Vet. Parasitol. 125, 163-181.

Fournier, D., Mutero, A., 1994. Modification of acetylcholinesterase as a mechanism of resistance to insecticides. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicol. and Endocrinology 108, 19-31.

Fragoso, S.H., Soberanes, C.N. 2001. Control de la resistencia a los ixodicidas a la luz de los conocimientos actuales. In Memorias de XXV Congreso Nacional de Buiatría Veracruz.Veracruz, México.Asociación Mexicana de Médicos especialistas en Bovinos, A.C, pp. 40-48.

George, J.E., Pound, J.M., Davey, R.B., 2004. Chemical control of ticks on cattle and the resistance of these parasites to acaricides. Parasitol. 129 Suppl, S353-366.

Giri, S., Giri, A., Sharma, G.D., Prasad, S.B., 2002. Mutagenic effects of carbosulfan, a carbamate pesticide. Mutat. Res. 519, 75-82.

Gonzalez-Cid, M., Loria, D., Matos, E., 1990. Genotoxicity of the pesticide propoxur and its nitroso derivative, NO-propoxur, on human lymphocytes in vitro. Mutat. Res. 232, 45-48.

Guillet, P., N'Guessan, R., Darriet, F., Traore-Lamizana, M., Chandre, F., Carnevale, P., 2001. Combined pyrethroid and carbamate 'two-in-one' treated mosquito nets: field efficacy against pyrethroid-resistant *Anopheles gambiae* and *Culex quinquefasciatus*. Med. Vet. Entomol. 15, 105-112.

Gupta, R.C. 2006. Toxicology of organophosphate & carbamate compounds. USA. Elsevier.

Hanser, E., Mehlhorn, H., Hoeben, D., Vlaminck, K., 2002. In vitro studies on the effects of flubendazole against *Toxocara canis* and *Ascaris suum*. Parasitol. Res. 89, 63-74.

He, H., Chen, A.C., Davey, R.B., Ivie, G.W., George, J.E., 1999. Identification of a point mutation in the para-type sodium channel gene from a pyrethroid-resistant cattle tick. Biochem. Biophys. Res. Commun. 261, 558-561.

Hemingway, J., Hawkes, N.J., McCarroll, L., Ranson, H., 2004. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem.Mol. Biol. 34, 653-665.

Hubner, P., Groux, P.M., Weibel, B., Sengstag, C., Horlbeck, J., Leong-Morgenthaler, P.M., Luthy, J., 1997. Genotoxicity of ethyl carbamate (urethane) in *Salmonella*, yeast and human lymphoblastoid cells. Mutat. Res. 390, 11-19.

Jamroz, R.C., Guerrero, F.D., Pruett, J.H., Oehler, D.D., Miller, R.J., 2000. Molecular and biochemical survey of acaricide resistance mechanisms in larvae from Mexican strains of the southern cattle tick, *Boophilus microplus*. J. Insect Physiol. 46, 685-695.

Jiménez-Cardoso, E., Flores-Luna, A., Pérez-Urizar, J., 2004. In vitro activity of two phenyl-carbamate derivatives, singly and in combination with albendazole against albendazole-resistant *Giardia intestinalis*. Acta Tropica 92, 237-244.

Jongejan, F., Uilenberg, G., 2004, The global importance of ticks. Parasitology 129 Suppl, S3-14.

Katiyar, S., Gordon, V., McLaughlin, G., Edlind, T., 1994. Antiprotozoal activities of benzimidazoles and correlations with beta-tubulin sequence. Antimicrobial and chem. 38, 2086-2090.

Kevekordes, S., Gebel, T., Pav, K., Edenharder, R., Dunkelberg, H., 1996. Genotoxicity of selected pesticides in the mouse bone-marrow micronucleus test and in the sister-chromatid exchange test with human lymphocytes in vitro. Toxicol. Letters 89, 35-42.

Miller, R.J., Almazan, C., Ortiz-Estrada, M., Davey, R.B., George, J.E., De Leon, A.P., 2013. First report of fipronil resistance in *Rhipicephalus* (*Boophilus*) *microplus* of Mexico. Vet. Parasitol. 191, 97-101

Mutero, A., Pralavorio, M., Bride, J.M., Fournier, D., 1994, Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. Proc. Natl.Acad. Sci. U. S. A. 91, 5922-5926.

NOM-019-ZOO-1994, Campaña Nacional contra la *Garrapata Boophilus* spp. Recuperado en Agosto de 2011 de www.normateca.sagarpa.gob.mx.

Odilón, A., 1993. Síntesis de carbamatos a partir de aminas aromáticas. Tesis de Licenciatura. UNAM, México.

OECD, 2001. Guideline for testing of chemicals. Acute Oral Toxicity-Fixed Dose Procedure. Guideline 420.

Oliveira, M.C., Oliveira-Sequeira, T.C., Araujo, J.P., Jr., Amarante, A.F., Oliveira, H.N., 2005. Babesia spp. infection in *Boophilus microplus* engorged females and eggs in Sao Paulo State, Brazil. Vet. Parasitol. 130, 61-67.

Ordaz-Pichardo, C., Shibayama, M., Villa-Treviño, S., Arriaga-Alba, M., Angeles, E., de la Garza, M., 2005. Antiamoebic and toxicity studies of a carbamic acid derivative and its therapeutic effect in a hamster model of hepatic amoebiasis. Antimicrobial agents and chemotherapy 49, 1160-1168.

Orme, S., Kegley, S., 2003. PAN Pesticide Database, Pesticide Action Network, North America, San Francisco, CA. 2003. Disponible en Internet En: http: www. pesticideinfo. org.

Ortiz, E., 1999. El uso de ixodicidas en México. En: Segundo Seminario internacional de Parasitología animal: Garrapatas y enfermedades que transmiten, 57-65. Oaxtepec, Morelos, México.

Ortiz, E., Santamaría, V., Ortiz, N., Soberanes, C., Osorio, M., Franco, B., 1995. Purificación y caracterización toxicológica de garrapatas Boophilus microplus resistentes a ixodicidas organofosforados y orga-noclorados. VII Reunión Anual Asc. Mex. de Parasitología Veterinaria, A.C. Ciudad Victoria, Tamps. México.

Ozden, S., Catalgol, B., Gezginci-Oktayoglu, S., Arda-Pirincci, P., Bolkent, S., Alpertunga, B., 2009. Methiocarb-induced oxidative damage following

subacute exposure and the protective effects of vitamin E and taurine in rats. Food Chem. Toxicol. 47, 1676-1684.

Perez-Cogollo, L.C., Rodriguez-Vivas, R.I., Ramirez-Cruz, G.T., Miller, R.J., 2010, First report of the cattle tick *Rhipicephalus microplus* resistant to ivermectin in Mexico. Vet Parasitol 168, 165-169.

Pérez-González, I.E., Prado-Ochoa, M.G., Muñoz-Guzmán, M.A., Vázquez-Valadez, V.H., Velázquez-Sánchez, A.M., Avila-Suárez, B.L., Cuenca-Verde, C., Angeles, E., Alba-Hurtado, F., 2013. Effect of new ethyl and methyl carbamates on *Rhipicephalus microplus* larvae and adult ticks resistant to conventional ixodicides. Vet. Parasitol. http://dx.doi.org/10.1016/j.vetpar.2013.07.042.

Prado-Ochoa, M.G., Muñoz-Guzmán, M.A., Abrego-Reyes, V.H., Velázquez-Sánchez, A.M., Lara-Rocha, M., Cuenca-Verde, C., Angeles, E., Alba-Hurtado, F., 2013, Effect of new ethyl and methyl carbamates on biological parameters and reproduction of the cattle tick *Rhipicephalus microplus*. Vet. Parasitol. 194, 49-57.

Rajput, Z.I., Hu, S.H., Chen, W.J., Arijo, A.G., Xiao, C.W., 2006. Importance of ticks and their chemical and immunological control in livestock. J Zhejiang Univ. Sci. B. 7, 912-921.

Reyes-González, R., 2007. Evaluación de la actividad antibiótica del compuesto LQM 996 en *Meriones unguiculatus* infectado con *Helicobacter pylori*. Tesis de Licenciatura. UNAM, México.

Rodríguez-Vivas, R., Arieta-Román, R., Pérez-Cogollo, L., Rosado-Aguilar, J., Ramírez-Cruz, G., Basto-Estrella, G., 2010. Uso de lactonas macrocíclicas para el control de la garrapata *Rhipicephalus (Boophilus) microplus* en el ganado bovino. Archivos de medicina veterinaria 42, 115-123.

Rodriguez-Vivas, R.I., Quinones-Avila, F.J., Ramirez-Cruz, G.T., Cruz, D., Wagner, G., 2007ª. Isolation of a field strain of *Babesia bigemina* (*Piroplasma: Babesiidae*) and establishment of in vitro culture for antigen production. Rev Biol. Trop. 55, 127-133.

Rodriguez-Vivas, R.I., Rivas, A.L., Chowell, G., Fragoso, S.H., Rosario, C.R., Garcia, Z., Smith, S.D., Williams, J.J., Schwager, S.J., 2007b. Spatial

distribution of acaricide profiles (*Boophilus microplus* strains susceptible or resistant to acaricides) in southeastern Mexico. Vet. Parasitol. 146, 158-169.

Rodríguez-Vivas, R.I., E Hodgkinson, J., J Trees, A., 2012. Resistencia a los acaricidas en *Rhipicephalus* (*Boophilus) microplus*: situación actual y mecanismos de resistencia. Revista Mexicana de Ciencias Pecuarias 3, 9 a 24.

Rosado-Aguilar, J.A., Rodriguez-Vivas, R.I., Garcia-Vazquez, Z., Fragoso-Sanchez, H., Ortiz-Najera, A., Rosario-Cruz, R., 2008. Development of amitraz resistance in field populations of *Boophilus microplus* (Acari: Ixodidae) undergoing typical amitraz exposure in the Mexican tropics. Vet. Parasitol. 152, 349-353.

Rosario-Cruz, R., Almazan, C., Miller, R.J., Dominguez-Garcia, D.I., Hernandez-Ortiz, R., de la Fuente, J., 2009. Genetic basis and impact of tick acaricide resistance. Front. Biosci. 14, 2657-2665.

Shafer, T., Meyer, D.A., Crofton, K.M., 2005. Developmental neurotoxicity of pyrethroid insecticides: Critical review and future research needs. Environ. Health. Perspect. 13, 123-136.

Schuntner, C. A., and Thompson P. G.. 1976**.** Inhibition of a carbaryl oxidising enzyme as the primary lesion in the lethal action of formamidines in *Boophilus microplus.* J. Aus. Entomol. Soc. 15: 388.

Seth, V., Banerjee, B.D., Bhattacharya, A., Chakravorty, A.K., 2000. Lipid peroxidation, antioxidant enzymes, and glutathione redox system in blood of human poisoning with propoxur. Clin. Biochem. 33, 683-685.

Soberanes-Céspedes, N., Santamaría-Vargas, M., Sánchez-Fragoso, H., García-Vázquez, Z., 2002. Primer caso de resistencia al amitraz en la garrapata del ganado *Boophilus microplus* en México. Téc. Pecu. Méx. 40, 81-92.

Sogorb, M.A., Vilanova, E., 2002. Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. Toxicol. Lett. 128, 215-228.

Solorio-Rivera, J.L., Rodriguez-Vivas, R.I., Perez-Gutierrez, E., Wagner, G., 1999. Management factors associated with *Babesia bovis* seroprevalence in cattle from eastern Yucatan, Mexico. Prev. Vet. Med. 40, 261-269.

Soulsby, E. 1987. Parasitología y enfermedades parasitarias. México: Ed Interamericana.

Soulsby, E., 1988. Parasitología y enfermedades parasitarias en los animales domésticos, EJL Soulsby, Helminths, arthropods and protozoa of domesticated animals.

Stone, B., Haydock, K., 1962. A method for measuring the acaricide-susceptibility of the cattle tick *Boophilus microplus* (Can.). Bull. Entomo. Res. 53, 563-578.

Tan, J., Liu, Z., Wang, R., Huang, Z.Y., Chen, A.C, Gurevitz, M., Dong, K. 2005. Identífication of amino acid residues in the insects sodium channel critical for pirethroid binding. Mol. Pharmacol. 67, 513-522.

Tan, F., Wang, L., Wang, J., Wu, X., Zhu, H., Jiang, L., Tao, S., Zhao, K., Yang, Y., Tang, X., 2011. Enhanced pesticide sensitivity of novel housefly acetylcholinesterases: a new tool for the detection of residual pesticide contamination. Bioprocess Biosyst. Eng. 34, 305-314.

Temeyer, K.B., Pruett, J.H., Untalan, P.M., Chen, A.C., 2006. Baculovirus expression of BmAChE3, a cDNA encoding an acetylcholinesterase of *Boophilus microplus* (Acari: Ixodidae). J. Med. Entomol. 43, 707-712.

Topaktas, M., Rencuzogullari, E., Ila, H.B., 1996. In vivo chromosomal aberrations in bone marrow cells of rats treated with Marshal. Mutat. Res. 371, 259-264.

WHO (World Health Organization) Expert Committee on Insecticides., 1957. Seventh report, Geneva, World Health Organization. Technical Report Series No. 125