UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (BIOLOGIA CELULAR E MOLECULAR)

ALTERAÇÕES MORFOFISIOLÓGICAS DO TEGUMENTO DE COELHOS INFESTADOS POR CARRAPATOS *Rhipicephalus sanguineus* (ACARI: IXODIDAE) E EXPOSTOS À SELAMECTINA (PRINCÍPIO ATIVO DO ACARICIDA REVOLUTION®, PFIZER)

VLAMIR BOZZATTO DE OLIVEIRA

Dissertação apresentada ao Instituto de Biociências do campus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas (Biologia Celular e Molecular).

Maio/2013

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“Said it all

Nothing to say it all

Nothing to say that matters

Haven't we heard enough?”

(Take That – Said It All)

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RESUMO

Carrapatos são ectoparasitas obrigatoriamente hematófagos que podem transmitir diversas doenças aos hospedeiros durante o seu processo de alimentação. Ao lesionar mecanicamente o tecido acabam induzindo respostas inflamatórias na pele, local onde se fixam. Na tentativa de minimizar a ação destes sobre os hospedeiros tem-se recorrido ao controle químico por meio da utilização de diversos produtos, dentre estes a selamectina, princípio ativo de muitos acaricidas, inclusive do Revolution® (Pfizer), uma lactona macrocíclica capaz de causar danos neurotóxicos no carrapato. Foi objetivo deste trabalho analisar, por meio de técnicas histológica, histoquímica, detecção da atividade da fosfatase ácida (enzimas hidrolíticas responsáveis pela detecção de morte celular autofágica) e microscopia confocal de varredura a laser, a ocorrência de alterações morfofisiológicas na pele de coelhos hospedeiros expostos à selamectina e infestados por *Rhipicephalus sanguineus* adultos. Os coelhos (n=12) foram expostos ao produto nas concentrações de 100% (grupo de tratamento I – **GTI**) e de 80% (grupo de tratamento II – **GTII**) e posteriormente infestados apresentaram no exame histológico diminuição da espessura da camada córnea do epitélio, o qual sofreu diminuição da quantidade de camadas celulares (adelgaçamento), processo este acompanhado pela formação acentuada de edemas subepidérmicos (com aumento de exudato), o que provocou a desorganização das fibras colágenas do tecido conjuntivo da camada dérmica. Os testes histoquímicos revelaram forte marcação PAS positiva nos folículos pilosos e em algumas regiões da derme e ressíntese de fibras colágenas demonstrada por meio da técnica do tricômico de Mallory. Os resultados obtidos para a detecção da atividade da fosfatase ácida nos indivíduos expostos às concentrações de 100% e 80% de selamectina mostraram forte marcação da fosfatase ácida no epitélio, nas células do tecido conjuntivo da derme e nos folículos pilosos. As imagens confocais corroboraram os resultados das análises histológicas, evidenciando o adelgaçamento do epitélio da epiderme dos indivíduos dos grupos de tratamento I e II (**GTI** e **GTII**), sendo possível visualizar monoestratificação desta camada em algumas regiões nos indivíduos do grupo de tratamento I (**GTI**). Os indivíduos expostos à concentração de 50% (grupo de tratamento III – **GTII**) apresentaram, em todas as análises aqui realizadas, respostas morfofisiológicas semelhantes àquelas encontradas nos do grupo controle. Todos os resultados aqui obtidos mostraram que a selamectina agiu como agente tóxico quando em contato com a pele dos coelhos infestados por carrapatos, alterando morfofisiologicamente o processo de inflamação aguda no tegumento destes animais. A selamectina é uma substância química com ação dose-dependente, e quanto maior foi a concentração, maiores foram os danos morfofisiológicos provocados na pele dos coelhos leporídeos. Quando utilizada em pequenas doses (menores do que as indicadas pelo fabricante), esse composto ainda é capaz de eliminar os carrapatos porém sem causar grandes danos no tegumento dos hospedeiros (organismos não-alvo).

Palavras-chave: pele, resposta imuno-inflamatória, infestação, fosfatase ácida, toxicologia.

Abstract

ABSTRACT

Ticks are ectoparasites obligatorily hematophagous that can transmit various diseases to their hosts during the feeding process. By mechanically injured in the tissue eventually inducing inflammatory responses in the skin where they attach. In an attempt to minimize their action on the host has recourse to chemical control through the use of various acaricides, among these selamectin, the active principle of many acaricides, including the Revolution ® (Pfizer), a macrocyclic lactone capable of causing neurotoxic damage. This study aimed to analyze, using histological techniques, histochemical, detection of acid phosphatase activity (hydrolytic enzymes responsible for the detection of autophagic cell death) and confocal laser scanning, the occurrence of morphophysiological changes in the skin of hosts rabbits exposed to the selamectin and infested by adults of *Rhipicephalus sanguineus*. The rabbits (n=12) exposed to the product at concentration of 100% (treatment group I - **TGI**) and 80% (treatment group II - **TGII**) and infested showed, in histologic examination, a partial and/or complete decrease in the *stratum corneum* of epithelium, which suffered a decrease in the amount of cell layers with consequent reduction of stratification (thinning) of the epithelium and marked subepidermal edema formation (with increase of exudates) which caused the disorganization of the collagen fibers of the connective tissue of the dermal layer. Histochemical tests showed strong staining PAS positive in hair follicles and in some regions of the dermis, and the resynthesis of collagen fibers has been demonstrated by Mallory trichrome technique. The confocal images corroborate the results of the histological analysis, showing the thinning of the epithelium of the individuals' skin of the treatment groups I and II (**TGI** and **TGII**), where this layer can view monostratification in some regions in individuals in the treatment group I (**TGI**). Individuals exposed to a concentration of 50% (treatment group III - **TGII**) showed, in all analyzes performed here, morphophysiological responses similar to those found in the control group. All obtained results showed that selamectin acted as a toxic agent when in contact with the skin of rabbits infested by ticks, changing morphophysiologically the process of acute inflammation in the tegument of these animals. Selamectin is a chemical substance which has a dose-dependent action, since higher the concentration, greater is the morphophysiological damage found in the skin of rabbits. When used in small doses (less than are indicated by the manufacturer), this compound is also able to eliminate the ticks without causing major damage to the tegument of the host (non-target organisms).

Key words: skin, immune-inflammatory response, infestation, acid phosphatase, toxicology.

Introdução Geral

1. INTRODUÇÃO GERAL

Os carrapatos, animais considerados de grande importância médico-veterinária, são artrópodos pertencentes à classe Arachnida, ordem Acari, tendo como principais famílias a Ixodidae e a Argasidae (MASSARD; FONSECA, 2004). Distinguem-se facilmente dos insetos por apresentarem, de maneira geral, quatro pares de patas nos estágios de ninfa e adulto (RUPPERT et al., 2005).

O corpo dos ácaros não apresenta segmentação, sendo, portanto, constituído de apenas uma peça que na região anterior é denominada gnatossoma ou capítulo e está dotada de estruturas cortantes denominadas quelíceras, além de apresentar o hipostômio e os palpos. Posteriormente ao gnatossoma, existe o idiossoma onde alojam-se os quatro pares de pernas (FLECHTMANN, 1985).

Todas as espécies de carrapatos são obrigatoriamente hematófagas, alimentando-se do sangue de outros animais. Possuem significativo grau de especificidade, podendo se fixar em diferentes hospedeiros: roedores, lagomorfos, marsupiais, carnívoros, cervídeos, répteis, aves e potencialmente no homem (MASSARD; FONSECA, 2004). Ácaros hematófagos do tipo mesostigmata (que inclui parasitos de vertebrados e vetores de potenciais agentes patogênicos) são ovíparos e com distribuição cosmopolita (FLECHTMANN, 1985).

Os mamíferos cenozóicos formam um grupo altamente diversificado de organismos, adaptados a uma ampla variedade de estilos de vida e apresentando grande diversidade ecomorfológica (POUGH et al., 2003). A classe Mammalia, cosmopolita, apresenta cerca de 5.400 espécies distribuídas em aproximadamente 46 ordens (WILSON; REEDER, 2005).

Os coelhos do gênero *Oryctolagus* são mamíferos pertencentes à ordem Lagomorpha, família dos Leporídeos, espécie *Oryctolagus cunículos* (PINHEIRO JÚNIOR, 1973). Os coelhos utilizados em trabalhos científicos são tipicamente coelhos New Zeland White, os quais apresentam pelos brancos e padrão de coloração avermelhada dos olhos.

O revestimento externo do corpo destes mamíferos é composto pela pele e suas especializações, a qual tem como função proteger o corpo da exposição e das contínuas mudanças que ocorrem no meio externo, enquanto, concomitantemente, promove a interação com o meio interno do corpo e suas flutuações fisiológicas. Além disso, a pele contribui com a percepção sensorial externa, termorregulação e defesa imunológica do indivíduo (SAMUELSON, 2007).

A pele é composta de duas regiões distintas: a epiderme e a derme. A epiderme possui externamente um epitélio estratificado pavimentoso, geralmente queratinizado. Logo abaixo da epiderme, existe a derme, composta por tecido conjuntivo denso não modelado que

abriga vasos sanguíneos, nervos, glândulas, entre outras estruturas (JUNQUEIRA; CARNEIRO, 2009). O desenvolvimento de ambas as regiões varia consideravelmente de espécie para espécie, dependendo da necessidade de proteção do animal contra dessecação e abrasão (SAMUELSON, 2007).

Além da epiderme e da derme, encontra-se uma outra estrutura associada à pele – a hipoderme, situada abaixo da derme. A hipoderme, também chamada de tecido subcutâneo, é rica em tecidos adiposo e conjuntivo e serve para ancorar a pele aos músculos (JUNQUEIRA; CARNEIRO, 2009).

Durante o processo de alimentação, o aparelho bucal dos carrapatos penetra na pele do hospedeiro, o que possibilita sua fixação por meio do hipostômio (na região central da cabeça), processo auxiliado pela solidificação da secreção salivar (cone de cemento) destes ectoparasitas. Ao provocar laceração dos tecidos e dos vasos sanguíneos, os carrapatos conseguem ingerir o sangue e outros líquidos tissulares dos hospedeiros sendo que regurgitam grandes volumes de saliva, a qual contém anticoagulantes e é concomitantemente a principal via de inoculação de diversos patógenos (MASSARD; FONSECA, 2004).

No ambiente rural brasileiro e na periferia das áreas urbanas é comum a presença de cães parasitados pelo carrapato *Rhipicephalus sanguineus* (LATREILLE, 1806) (Acari: Ixodidae). Popularmente conhecido como carrapato vermelho, se caracteriza pela hematofagia em animais de sangue quente, tendo como hospedeiro preferencial o cão, mas podendo parasitar cavalos e bovinos e até mesmo o homem. Seu ciclo biológico envolve hospedeiros e suas fêmeas colocam aproximadamente 4.000 ovos (LABRUNA; PEREIRA, 2001). Estes animais passam por quatro estágios de vida, a saber: ovo, larva, ninfa e adulto. Assim que a larva eclode do ovo, esta fixa-se no primeiro hospedeiro e se alimenta por aproximadamente quatro dias, quando então se destaca e sofre ecdise para o próximo estágio, a ninfa. Esta, por sua vez, repete o ciclo de quatro dias fixada ao segundo hospedeiro, sofre nova ecdise no solo e transforma-se em adulto (machos e fêmeas) que se alimentam no terceiro hospedeiro por aproximadamente sete dias, copulam e, após terminado o período de alimentação, as fêmeas caem no solo, ovipõem e morrem em seguida (LABRUNA; PEREIRA, 2001).

O carrapato *R. sanguineus* tem tido cada vez mais um papel relevante entre as espécies de carrapatos de importância mundial. Nos cães, além dos danos diretos, ele causa a anemia por infestação (JERNIGAN et al., 2000), e é também responsável pela transmissão de *Ehrlichia canis*, *Babesia canis*, *Haemobartonella canis* e *Hepatozoon canis* (DANTAS-TORRES, 2010). Nos humanos, esta espécie ainda é vetora da bactéria *Rickettsia conorii* na

Europa e da *R. rickettsii* no Arizona – EUA (BORGES et al., 2007). No Brasil existem relatos de parasitismo humano por este carrapato (DANTAS-TORRES et al., 2006).

Por sua importância epidemiológica e econômica, o controle de carrapatos têm sido objeto de estudos, mas apesar do avanço da ciência, até o presente momento as infestações de carrapatos tem sido controladas por acaricidas químicos, que embora eliminem os ectoparasitas, trazem prejuízos aos organismos não alvos, bem como ao ambiente (KIM; FEAGLEY, 1998; POPPENGA; OEHME, 2010).

Exposições agudas a pesticidas podem ser suficientes para produzir sinais clínicos e resultados indesejados como envenenamento agudo, indicando toxicidade e até mesmo a liberação de resíduos que afetam diretamente a segurança pública por meio da contaminação da cadeia alimentar. O uso de pesticidas pode afetar indiretamente a vida selvagem tendo como resultado a eliminação de algumas espécies que desempenham importante papel na cadeia alimentar (POPPENGA; OEHME, 2010).

Carrapaticidas como as avermectinas são importantes anti-parasitários, devido ao seu amplo espectro de ação, à alta eficiência, à margem de segurança favorável e por possuir apenas um mecanismo de ação, ou seja, aquele que provoca a paralisia do ectoparasita através da abertura dos canais de íons cloro da membrana das células do sistema nervoso periférico, ocasionando a desestruturação no potencial de ação de toda membrana celular (NOVOTNY et al., 2000). A tolerância a esta classe de drogas é diferente entre as espécies de mamíferos. Assim, é extremamente importante demonstrar a segurança de uma avermectina específica nas espécies em geral antes de utilizar a sua atividade reforçada como antiparasitário (KRAUTMANN et al., 2000).

A selamectina, uma subclasse das avermectinas, é uma lactona macrocíclica muito utilizada no controle de ectoparasitas de cães e gatos domésticos, e que mostra-se eficiente na sua eliminação (KRAUTMANN et al., 2000; NOVOTNY et al., 2000). Embora as lactonas macrocíclicas possuam ampla margem de segurança, intoxicações já foram constatadas em diversos estudos com cães (HOPKINS et al., 1990; HADRICK et al., 1995; BEAL et al., 1999; HOPPER et al., 2002; SNOWDEN et al., 2006; KENNY et al., 2008). Collies e raças semelhantes podem desenvolver intoxicação devido à expressão limitada da atividade da p-glicoproteína na barreira hematoencefálica (HOOPER et al., 2002). Existem diversos casos de intoxicação em cães atribuídos à ingestão de doses administradas em cavalos (HOPKINS et al., 1990; BEAL et al., 1999; SNOWDEN et al., 2008).

A selamectina é distribuída via corrente sanguínea, acumulando-se principalmente nas glândulas sebáceas da pele, onde forma reservatórios contra pulgas, carrapatos e ácaros do

pavilhão auricular (HOVDA; HOOSER, 2002). Dentre os principais acaricidas que possuem como principal composto a selamectina, destaca-se o Revolution®, fabricado pelo laboratório Pfizer.

Estudos sobre dosagens do produto químico realizadas em gatos (via oral) resultaram em salivação anormal e emese intermitente, indicando potencial toxicidade da selamectina (PFIZER, 1999; KRAUTMANN et al., 2000). Dentre os efeitos clínicos encontrados, pode-se citar: irritação da pele, alopécia transitória, aglutinação e descoloração de pêlos e até mesmo a persistência de resíduos (em forma de pó branco) do produto químico no local do tratamento (NOVOTNY et al., 2000; HOVDA; HOOSER, 2002). De acordo com Hovda; Hooser (2002), vômitos, diarréia, anorexia, inércia, taquipnéia e tremores musculares também foram observados em alguns casos após exposição à selamectina.

Apesar de se conhecer a ação acaricida (neurotóxica) da selamectina no organismo do carrapato e de haver trabalhos relacionados à efetividade deste produto quanto à morte do ectoparasita, justifica-se a realização do presente estudo: a) pela ampla utilização da selamectina como acaricida em cães e gatos domésticos, portanto, atingindo não só os organismos alvo, mas também os não alvos, bem como o ambiente; b) a necessidade de controle e eliminação dos ectoparasitas por meio de drogas e suas concentrações que não sejam perigosas nem agressivas aos hospedeiros e ao ambiente, e no caso da selamectina, por esta provocar reações adversas no local de tratamento, tais como: irritação, perda e descoloração de pêlos e persistência de resíduos em pó (KRAUTMANN et al., 2000; NOVOTNY et al., 2000; HOVDA; HOOSER, 2002). A bula do produto recomenda que os usuários devem evitar o contato com o produto, via proteção dos dedos durante a sua aplicação (PFIZER SAÚDE ANIMAL, 2012).

Objetivos

2. OBJETIVOS

A partir do exposto, buscando agregar informações que possibilitem o aumento da eficiência do controle de carrapatos *Rhipicephalus sanguineus* com o mínimo de impacto para os hospedeiros, o presente trabalho teve como objetivo avaliar, por meio de análises histológicas, histoquímicas, técnica para a detecção da atividade da fosfatase ácida (enzimas hidrolíticas responsáveis pela detecção de morte celular autofágica) e análise morfológica por meio de microscopia confocal de varredura a laser quais são e de que tipo são as alterações que ocorrem no tegumento dos coelhos pré-infestados com *R. sanguineus* após a aplicação das diferentes concentrações de selamectina.

Com isso, pretendeu-se demonstrar também se a lesão que normalmente é formada no tegumento dos hospedeiros pela picada do carrapato está sendo alterada ou não com a presença da selamectina, modificando, consequentemente, a morfologia e/ou fisiologia do tegumento no local da picada.

Material e Métodos

3. MATERIAL E MÉTODOS

3.1 Material

3.1.1 Local do Estudo

Para a realização do presente estudo foram utilizados equipamentos disponíveis nas dependências dos laboratórios de Histologia e Microscopia Eletrônica do Departamento de Biologia do I.B.- UNESP- Campus de Rio Claro, SP. Nesta Instituição já está implantada e encontra-se em pleno funcionamento a Brazilian Central of Studies on Ticks Morphology (BCSTM), onde colônias de carrapatos *Rhipicephalus sanguineus* são mantidas em leporídeos e são assistidas e utilizadas por um grupo de 20 pesquisadores, sob a coordenação da Profa. Dra. Maria Izabel Camargo Mathias.

Este projeto foi submetido para análise pelo Comitê de Ética em Pesquisa no Uso Animal da UNESP – Rio Claro, SP (CEUA – IB – UNESP – CRC) e obteve aprovação com o protocolo n° 9058.

3.1.2 Substância Química: Selamectina (Revolution™ 12%).

A selamectina foi obtida a partir do acaricida Revolution™ 12%, Pfizer Saúde Animal, em tubos de 0,25 mL, concentração de 30mg/mL, para animais de 2,6 a 5,0 Kg. O grupo de hospedeiros que receberam o tratamento foi submetido a diferentes concentrações de selamectina, sendo estas a própria dose de 100% obtida no produto e as diluições do produto em água destilada a 80% e 50%.

3.1.3 Carrapatos

Para o presente estudo foram utilizados casais de carrapatos adultos semi-ingurgitados de *R. sanguineus*, coletados a partir de colônia mantida em condições controladas (28º C, 85% de umidade e fotoperíodo de 12 h) em estufa BOD, em sala de Biotério do Departamento de Biologia (IB) – UNESP, campus de Rio Claro/SP, Brasil.

3.1.4 Hospedeiros

Para a realização deste trabalho foram utilizados 12 coelhas fêmeas (New Zealand White) saudáveis, com peso semelhante (± 3,5 Kg) e provenientes do Biotério Central da UNESP, campus de Botucatu, SP, que foram mantidas em gaiolas de contenção e alimentadas com ração apropriada e água *ad libitum* no Biotério da UNESP – Campus de Rio Claro/SP – Brasil.

A infestação artificial desses animais, a confecção e fixação das câmaras de alimentação, bem como a liberação dos carrapatos dentro destas seguiu os procedimentos descritos por Bechara et al. (1995).

3.1.5 Modelo Experimental

Para o desenvolvimento deste projeto foram estabelecidos quatro grupos de estudo:

Grupo I (GC)	**Grupo II (GTI)**	**Grupo III (GTII)**	**Grupo IV (GTIII)**
3 coelhas livres da exposição ao produto Revolution®	3 coelhas expostas a selamectina 100%	3 coelhas expostas a selamectina 80%	3 coelhas expostas a selamectina 50%

–Grupo Controle (GC)

Os hospedeiros deste grupo não foram expostos ao produto. Nestes foram depositados 25 casais de carrapatos adultos em jejum, segundo método descrito por Bechara et al. (1995). Após três dias de ingurgitamento as fêmeas foram removidas. A região do tegumento das coelhas onde os carrapatos ficaram fixados juntamente com o hipostômio dos mesmos foi retirada (via biópsia) e colocada em fixador adequado para processamento.

–Biópsia da Pele dos Hospedeiros

Foram coletadas amostras de pele dos hospedeiros (nos locais das picadas dos carrapatos) por meio de "punch" (saca-bocados) com 3,0 mm de diâmetro, local que foi previamente anestesiado com xilocaína 2% sem vaso-constritor, procedimento realizado pela veterinária Letícia Maria Gráballos Ferraz Hebling. O procedimento consistiu em estirar a pele das coelhas e introduzir o punch (manobra de Whyte e Perry) por meio de movimentos de rotação e pressão vertical, fazendo com que o instrumento atingisse a profundidade desejada (camada dérmica da pele).

–Grupos Tratamento (GTI, GTII e GTIII)

As coelhas dos grupos de tratamento (três coelhos/grupo) foram expostas ao produto, recebendo as doses de selamectina propostas anteriormente.

Os hospedeiros dos Grupos de Tratamento foram infestados artificialmente com 25 casais de carrapatos adultos em jejum, segundo Bechara et al. (1995). No primeiro dia após a infestação, receberam as doses de selamectina sob a forma "pour on" (aplicação sobre a linha dorsal do corpo). Após três dias de alimentação, todos os carrapatos foram encontrados

mortos (ARAUJO, comunicação pessoal), sendo as fêmeas removidas e a região dos hospedeiros onde os carrapatos ficaram fixados juntamente com o hipostômio dos mesmos retirada por meio de biópsia e todo o material obtido foi encaminhado para o laboratório para processamento.

3.2 Métodos

3.2.1 Histologia do tegumento das coelhas (Historesina)

Após a remoção dos carrapatos, os fragmentos de tegumento foram fixados por 24 horas em solução de paraformaldeído a 4% e NaCl a 0,9% em tampão fosfato 10% (0.1M - pH 7,5). A seguir, todo o material foi desidratado em soluções crescentes de etanol a 70, 80, 90 e 95% durante 15 minutos cada. Logo após, foram transferidos para solução de Resina (JB-4 Polaron Instruments/Bio Rad) na ausência de catalisador, durante 24 horas. Posteriormente, as amostras foram transferidas para moldes plásticos previamente preenchidos com resina contendo catalisador. Os moldes foram selados com suportes de madeira para microtomia. Depois de polimerizados os blocos foram seccionados com auxílio de Micrótomo Sorvall JB-4/Bio Rad. As secções de 3 μm de espessura foram hidratadas e recolhidas em lâminas de vidro. Depois de secas, as lâminas foram submetidas à coloração pela hematoxilina de Harris e eosina aquosa (HE) durante 10 e 5 minutos, respectivamente. Em seguida foram secas, diafanizadas em xilol e montadas em bálsamo do Canadá.

3.2.2 Técnica do Azul de Bromofenol para detecção de proteínas (Segundo Pearse, 1985) no tegumento das coelhas

Os fragmentos do tegumento dos hospedeiros foram fixados por 48 horas em paraformaldeído 4%. As secções foram coradas com Azul de Bromofenol por 2 horas em temperatura ambiente. Após, foram lavadas com ácido acético 0,5% por 5 minutos e com água corrente por 15 minutos e as mesmas passaram rapidamente por solução de álcool butílico terciário.

A seguir, foram secas ao ar livre, diafanizadas e montadas em bálsamo do Canadá.

3.2.3 Técnica simultânea de PAS e Alcian Blue para detecção de polissacarídeos neutros e ácidos (Segundo JUNQUEIRA; JUNQUEIRA, 1983) no tegumento das coelhas

Os fragmentos do tegumento de todos os hospedeiros foram fixados em Bouin aquoso por 48 horas. Depois de seccionados e colocados em lâminas, os mesmos foram lavados com água destilada, coradas com Alcian Blue (pH=2,5) por 30 minutos. Em seguida, foram

lavados rapidamente em água destilada e expostos ao ácido periódico 1% por 10 minutos. Novamente foi realizada lavagem em água destilada. A seguir as secções foram expostas ao reativo de Schiff durante 30 minutos, no escuro. Posteriormente, procedeu-se três lavagens em água sulfurosa (3 minutos cada) e, em seguida, as lâminas foram lavadas em água corrente por 30 minutos. Após secagem, as lâminas contendo as secções foram colocadas em xilol para diafanização e montadas com Bálsamo do Canadá.

3.2.4 Tricômico de Mallory para detecção de fibras colágenas (JUNQUEIRA; JUNQUEIRA, 1983) no tegumento dos coelhos

As lâminas contendo as secções histológicas foram colocadas em placas de Petri, onde se depositou lugol por 3 a 5 minutos e, então, solução de hipossulfito aquoso a 5% por mais 3 minutos. As lâminas foram então lavadas em água corrente por 20 minutos. Receberam hematoxilina por 10 minutos (reagindo com água), logo após coradas com o corante tricômico de Mallory por 15 minutos em estufa a 37°C, lavadas, desidratadas e montadas para exame microscópico.

3.2.5 Detecção da Atividade da Fosfatase Ácida (HUSSEIN et al., 1990) no tegumento das coelhas

Fragmentos do tegumento resultantes da biópsia nos hospedeiros foram fixados em formalina neutra tamponada 10% (pH 7,4) e acetona, na proporção de 9:1, durante 1 hora e 30 minutos, a 4ºC. Na seqüência foram lavados em tampão acetato de sódio (0,05M, pH 4,8) e incubados por 45 minutos a 37°C no meio: naftol AS-TR fosfato, DMSO (dimetil sulfoxido), tampão acetato de sódio (0,05M, pH 4,8), MnCl2.4H2O 10% e o sal fast red violet.

Para o preparo do meio de incubação foram dissolvidos 3 mg do substrato naftol AS-TR fosfato em duas gotas de DMSO e, em seguida, adicionados 10 mL de tampão acetato de sódio. Então foi acrescentado 0,2 mL de cloreto de manganês 10% mais 6 mg do sal fast red violet e, para finalizar, a solução final foi vigorosamente misturada. O controle foi realizado excluindo-se o substrato (3 mg de naphtol AS-TR fosfato) do meio de incubação.

O material foi desidratado em concentrações crescentes de álcool (70%, 80%, 90% e 95%), banhos de 15 minutos cada, incluído em parafina e seccionado. As secções de 7 µm foram recolhidas em lâminas de vidro e reidratadas por 1 minuto em água destilada, contracoradas por 1 minuto com hematoxilina de Harris, secas e montadas em Bálsamo do Canadá para observação ao microscópio de luz.

3.1.6. Microscopia confocal de varredura a laser da epiderme das coelhas hospedeiras

O tegumento das hospedeiras expostas a selamectina foi retirado e fixado em folmaldeído 3,4%. A seguir, o material foi permeado com 0,1% Triton X-100 por 20 minutos e por RNAase (10 mg/mL). Logo após, foi incubado "overnight" com mistura de anticorpos monoclonais anti-tubulina alfa e beta-tubulina (Sigma Aldrich) (1:100) no interior de câmara úmida. Após 3 lavagens com tampão fosfato-salino (PBS), as preparações foram incubadas por 1h com anticorpo secundário Cy5 anti-mouse (Molecular Probes) (1:100).

O material foi corado com faloidina-FITC (Sigma Aldrich) por 40 minutos e as preparações foram lavadas em PBS em cada etapa de cada reação. O material foi montado entre lâmina e lamínula com solução anti-fading (Vectashield, Vector) e contracorado utilizando 5 µL de iodeto de propídeo (PI) (10µ/mL).

As imagens foram obtidas com microscópio de varredura confocal a laser Leica TCS SP5-II e analisadas com Leica TCS SP5-II e software com Imaris (Bitplane).

Resultados

4. RESULTADOS

Os resultados obtidos no presente trabalho estão apresentados sob a forma de artigos científicos submetidos e/ou em análise em revistas internacionais.

Capítulo 1:

Situação: Submissão em Dezembro/2012 (Publicado online em Abril/2013).

Autores: BOZZATTO, V.[a], Oliveira, P.R.[a]; Camargo-Mathias, M.I. [a*]

Título: Histopathology of the tegument of rabbits infested by *Rhipicephalus sanguineus* (Acari: Ixodidae) ticks and exposed to selamectin (active principle of acaricide Revolution®, Pfizer).

Periódico: Parasitology Research.

Capítulo 2:

Situação: Submissão em Fevereiro/2013 (Pré-aceito em Março/2013).

Autores: BOZZATTO, V.[a], Oliveira, P.R.[a]; Furquim, K.C. [a]; Camargo-Mathias, M.I. [a*]

Título: The occurrence of autophagic cell death in the tegument of rabbits pre-infested with *Rhipicephalus sanguineus* and exposed to selamectin (Active principle of acaricide Revolution®, Pfizer)

Periódico: Microscopy Research and Technique.

Capítulo 3:

Situação: Submissão em Março/2013.

Autores: BOZZATTO, V.[a], Oliveira, P.R.[a]; Camargo-Mathias, M.I. [a*]

Título: Morphological alterations on rabbits epidermis infested by *Rhipicephalus sanguineus* ticks and exposed to selamectina (Active principle of acaricide Revolution®, Pfizer): Confocal microscopy

Periódico: Micron (Oxford).

Capítulo 1

HISTOPATHOLOGY OF THE TEGUMENT OF RABBITS INFESTED BY *Rhipicephalus sanguineus* (ACARI: IXODIDAE) TICKS AND EXPOSED TO SELAMECTIN (Active principle of acaricide Revolution®, Pfizer).

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RESUMO

Os carrapatos são ectoparasitas hematófagos que podem transmitir diversas doenças aos hospedeiros durante o processo de alimentação. Quando os carrapatos danificam mecanicamente os tecidos, podem eventualmente induzir respostas inflamatórias no local da pele onde estão fixados. Uma das alternativas para controlar estes ectoparasitas é o uso de substâncias químicas, como a selamectina – princípio ativo do acaricida Revolution®, Pfizer – uma lactona macrocíclica capaz de causar danos neurotóxicos no carrapato e eventualmente eliminar a infestação em cães e gatos. O objetivo deste estudo foi avaliar, por meio de técnicas histológicas e histoquímicas, a ocorrência de alterações morfofisiológicas na pele de coelhos hospedeiros expostos à selamectina e infestados com *Rhipicephalus sanguineus* (Acari: Ixodidae). Histologicamente, os coelhos expostos e infestados apresentaram diminuição parcial e/ou total da camada córnea, diminuição do número de camadas das células do epitélio, reduzindo consequentemente a estratificação (adelgaçamento) e formações evidentes de edemas subepidérmicos (com aumento de exudato) e consequente desorganização das fibras colágenas do tecido conjuntivo da camada dérmica. Os testes histoquímicos mostraram forte reação PAS positiva nos folículos pilosos e em algumas regiões da derme, além da ressíntese de fibras colágenas detectada pela técnica do tricrômico de Mallory. Os resultados obtidos mostraram que a selamectina age como um agente toxicante quando em contato com a pele dos coelhos infestados por carrapatos, induzindo alterações morfofisiológicas no processo inflamatório agudo no tegumento dos animais. A selamectina é uma substância química que tem ação dose dependente, uma vez que quanto maior a concentração, maiores são os danos morfofisiológicos encontrados na pele dos coelhos.

Palavras-chave: pele, toxicologia, morfofisiologia, inflamação.

ABSTRACT

Ticks are hematophagous ectoparasites which can transmit several diseases to the host during their feeding process. When ticks mechanically damage the tissue, they eventually induce inflammatory responses on the skin spot where they are fixed. One of the alternatives to control these ectoparasites is the use of chemical substances like selamectin – the active principle of Pfizer's antiparasitic Revolution® –, a macrocyclic lactone capable of doing neurotoxic damage to the tick and eventually eliminating infestation in dogs and cats. The purpose of this study was to analyze, by histological and histochemical techniques, the occurrence of morphophysiological alterations in the skin of the host rabbits exposed to

selamectin and infested with *Rhipicephalus sanguineus* (Acari: Ixodidae). Histologically, the exposed and infested rabbits showed a partial and/or total decrease in the *stratum corneum*, the epithelium decreased in the number of cell layers, consequently reducing the stratification (thinning) and quite pronounced formations of subepidermal edemas (with exudates) and consequent disorganization of collagen fibers in the dermal layer's connective tissue. The histochemical tests showed strong PAS positive reaction in the hair follicle and some regions of the dermis, besides the resynthesis of collagen fibers detected by the Mallory's trichrome technique. The obtained results showed that selamectin acts like a toxicant agent when in contact with the skin of the rabbit infested with ticks, inducing morphophysiological alterations in the acute inflammatory process in the animal's tegument. Selamectin is a chemical substance which has a dose-dependent action, since higher concentrations, cause greater morphophysiological damage in the skin of rabbits.
Key words: skin, toxicology, morphophysiology, inflammation.

1. INTRODUCTION

Ticks are inevitably bloodsucking ectoparasites that have a cosmopolitan distribution (Ribeiro et al. 1996) and are organisms advantageously resistant to environmental adversities (Walker 2000). Because of their feeding habits, ticks can transmit bacteria, protozoa, viruses and helminthes to domestic animals and humans, are considerate individuals of great importance to veterinary (Dantas-Torres 2010).

During the feeding process, the ticks' mouthparts penetrate deep into the host's skin, allowing their fixation through the hypostome (located in the central region of their head) aided by solidification of a salivary secretion (cement cone) (Balashov 1972). The laceration of tissues and blood vessels allows the ticks to take in blood with regurgitation of large amounts of saliva, which becomes the major way of inoculating pathogens (Massard and Fonseca 2004), besides being bioactive compounds capable of suppressing the hosts' immune responses, thereby facilitating the fixation process (Sauer et al. 1995; Meire et al. 2002; Bowman and Sauer 2004; De la Fuente et al. 2006). When ticks contact the hosts' skin, they mechanically damage their fixation spot (Massard and Fonseca 2004). According to Siqueira Junior and Dantas (2000), when aggression persists, tissues are damaged and it consequently leads to the development of an acute inflammatory response.

The inflammation is a process which may be defined as a reaction in the microcirculation induced in the tissue caused by injury, with the consequent movement of

intravascular elements such as fluids, cells and molecules into the extravascular space (Wilkinson et al. 1990; Siqueira Junior and Dantas 2000; Dos Santos et al. 2004).

For being epidemiological and economically importance, tick control has been the subject of many studies. However, despite the advancement of science, tick infestations on pets have so far only been controlled by chemical-based acaricides that kill ectoparasites and can be harmful to non-target organisms (hosts) and the environment (Kim and Feagley 1998; Blagburn and Dryden 2009; Rosado-Aguilar et al. 2010).

Acute exposures to pesticides may be large enough to produce clinical signs and undesirable outcomes such as acute poisoning (toxicity indicator), and even generate products that directly affect public health through contamination of the food chain. It can indirectly affect wildlife, resulting in the elimination of some species that play an important role in the food chain (Poppenga and Oehme 2010).

Selamectin, a subclass of avermectins, is a macrocyclic lactone widely used in ectoparasites control in domestic dogs and cats, which has been proven effective in eliminating these ectoparasites (Krautmann et al. 2000; Novotny et al. 2000). Although macrocyclic lactones have a wide safety margin for use in domestic animals, indicating that there have been no reports of human toxicity (according to the drug label), nevertheless, poisoning have been diagnosed in different studies with dogs (Hopkins et al. 1990; Hadrick et al. 1995; Beal et al. 1999; Hopper et al. 2002; Snowden et al. 2006; Kenny et al. 2008) and cats (Krautmann et al. 2000).

Despite knowing selamectin action (neurotoxic) in the organism of the ticks (Novotny et al. 2000), and that there are many studies related to the effectiveness of this product to kill ectoparasites (Krautmann et al. 2000), there are no studies on selamectin reporting the dynamic aspects of its implementation in hosts which are suffering acute inflammation processes induced by tick infestation. In this case, not only would the interaction of the acaricide with the inflammatory process in the host's skin be better understood, but it would also help to detect histopathological changes that selamectin might induce in these tissues, since this acaricide is generally administered to the pets when they already have many ticks attached to their bodies. Thus, the present study provides, through histological and histochemical techniques, information about tegument histophysiological changes in rabbits infested with *Rhipicephalus sanguineus* ticks, exposed to the selamectin action.

2. MATERIAL AND METHODS

2.1. Local Study

Equipment available in the facilities of the Laboratório de Histologia do Departamento de Biologia – IB – UNESP - Campus Rio Claro, SP, Brazil was used. This project was submitted to and reviewed by the Ethics Comission for Animal Use in Research at UNESP - Rio Claro, SP (CEUA - IB - UNESP - CRC) and forwarded to protocol No. 9058.

2.2. Chemical Substance: Selamectin (Revolution® 12%)

Selamectin was obtained from Revolution® 12% (Pfizer Animal Health), 30mg/mL concentration, for animals between 2.6 and 5.0 kg (5.7 and 11 pounds). The host groups receiving treatment were exposed to different concentrations of selamectin, here considerate 100% dose (the actual commercial product) and the product dilutions in distilled water at 80% and 50%.

2.3. Ticks

Rhipicephalus sanguineus semi-engorged adult couples were used. The couples were collected from a colony kept under controlled conditions (28 ºC, 85% humidity and 12 h photoperiod) in BOD incubator, in a room of the Biotério do Departamento de Biologia (IB) - UNESP, Rio Claro campus, SP, Brazil.

2.4 Hosts

Twelve healthy rabbits (New Zealand White) of a similar weight (± 3.5 kg), from the Biotério Central da UNESP, Botucatu campus, SP, Brazil were used. They were kept in cages, as well as appropriately fed and given *ad libitum* water, in a room of the Biotério da UNESP - Rio Claro, SP, Brazil.

Artificial infestation of the animals, construction and fixing of the feeding chamber, as well as putting the ticks into the animals inside the chamber, followed the procedures previously described by Bechara et al. (1995).

2.5. Experimental Model

- Control Group (CG)

The three hosts of this group were not exposed to the product. Twenty-five unfed adult tick couples were put on them, according to the method described by Bechara et al. (1995). After three days of engorgement, the females were removed. The region of the rabbit tegument, where ticks were fixed with the hypostome, was removed (by biopsy) and placed in a suitable fixer for subsequent histological and histochemical processing.

- Treatment Groups (TGI, TGII, TGIII)

The rabbits in the treatment groups were artificially infested with 25 fasting adult tick couples/rabbit, according to Bechara et al. (1995), and, after one day, exposed to the product.

The selamectin doses were applied based on the "pour on" form (application on the body topline), as indicated on Revolution label. (Pfizer Animal Health 2012). After three days of feeding the ticks are found dead (Araujo personal communication), the females were removed and the area where ticks were fixed on the hosts, together with their hypostome, was removed and biopsy material was taken for processing in the histology lab.

- Biopsy Specimen Collection of Hosts' Tissues

Skin samples were collected from the hosts (on the bite site) through punch with 3.0 mm diameter on the spot previously anesthetized with xylocaine 2% without vasoconstrictor, a procedure performed by veterinarian Leticia Maria Ferraz Gráballos Hebling. This technique is used to stretch the rabbit skin and insert the punch (Whyte and Perry maneuver) through rotation and vertical pressure, so that the instrument reaches the desired depth.

2.6. Methods

2.6.1. Histology and histochemistry of the tegument of rabbits

The tegument fragments were fixed for 24 hours in 4% paraformaldehyde, dehydrated in ethanol, embedded in Leica resin for 24 hours and transferred to plastic moulds previously filled with polymerized Leica resin. After resin polymerization**,** all the blocks were sectioned into 3 μm-thick slices on microtome Leica RM2245. Slides containing the sections were either stained with hematoxylin and eosin or forwarded for histochemical tests.

Histochemical tests were applied according to the bromophenol blue techniques to detect total proteins (Pearse 1985), in which the tegument fragments were fixed in 4% paraformoldehyde; the PAS simultaneous staining method (periodic acid-Schiff) and Alcian blue were applied to detect neutral and acidic polysaccharides (Junqueira and Junqueira 1983), in which the tegument fragments were fixed in aqueous Bouin; and the Mallory's trichrome technique was applied to detect collagen fibers (Junqueira and Junqueira 1983), in which the tegument fragments were fixed in 4% paraformoldehyde.

3. RESULTS

3.1. Histopathology of tegument

3.1.1 Control Group (CG)

Control group individuals had minor modifications resulting from penetration of hypostome for ticks to attach to the rabbit tegument, and among them are: a) hyperkeratosis (thickening of the *stratum corneum*) (Figs. 1A – 1C); b) epithelial hyperplasia (thickened epidermis) (dotted in Fig. 1A); c) subepidermal edema formation filled with leukocytes (Figs.

1A – 1C); d) leucocytes in the connective tissue of the dermal layer (Figs. 1A – 1C). Sebaceous glands (Fig. 1F) and sweat glands (Fig. 1G) were observed among the cells of the dermal connective tissue.

3.1.2. Treatment Group I (TGI)

Individuals exposed to selamectin 100% and infested with *R. sanguineus* ticks (Fig. 2C) showed the following histopathological changes: a) partial and/or total decrease in the *stratum corneum* (Fig. 2A – B); b) thinned epithelium with unistratification in some regions (dotted in Fig. 2A); c) a quite pronounced formation of subepidermal edemas (asterisks in Figs. 2A - C) with consequent disorganization of collagen fibers in the dermal layer connective tissue (Figs. 2A – B); d) migration of large amounts of leucocytes into the inflammatory infiltrate (Fig. 2A).

3.1.2. Treatment Group II (TGII)

Individuals exposed to selamectin 100% and infested with ticks (Fig. 3C) showed: a) a partial and/or total decrease in the *stratum corneum* (Figs. 3A – B); b) thinned epithelium (dotted in Figs. 3A – B); c) a minor subepidermal edema formation (asterisks in Figs. 3A - C) compared to the control group (**CG**), the presence of large amounts of leukocytes (red and white) in edemas (Fig. 3B).

3.1.3. Treatment Group III (TGIII)

Individuals exposed to selamectin 100% and infested with ticks (Fig. 4C) showed: a) a partial decrease in the *stratum corneum*; b) the formation of few edemas in the dermal connective tissue (asterisks in Figs. 4A - C) compared to the control group (**CG**); c) the presence of moderate amounts of leukocytes. The epithelium appeared to be regularly stratified, similar to that observed in the control group (GC) (dotted in Fig. 4A).

3.2. Histochemistry of tegument

3.2.1. Control Group (CG)

The *stratum corneum* is a layer that gets strongly stained by the bromophenol blue technique, indicating the presence of large amounts of total protein (Fig. 1D). However, it shows a moderate reaction for acidic polysaccharides and does not react with neutral polysaccharides (Fig. 1B). The cells of the epithelial layer are moderately stained by bromophenol blue (Fig. 1D), but the nuclei and cell boundaries are clearly evident. For

neutral polysaccharides, the reaction is weak and acidic polysaccharides are not detected in their constitution (Fig. 1B). The basal membrane is strongly PAS positive (Fig. 1B). The dermal connective tissue is strongly stained by the bromophenol blue technique (Fig. 1D); but for the polysaccharides, there are different reaction intensities (Fig. 1B). The presence of blood cells strongly stained by bromophenol blue is evident (Fig. 1D). Collagen fibers are seen in blue, whereas elastin fibers are seen in yellow in Mallory's trichrome.

Histochemical test results in the control group (**CG**) are summarized in Table 1.

3.2.2. Treatment Group I (TGI)

Individuals exposed to 100% selamectin of the dose recommended on Revolution® and previously infested with *R. sanguineus* ticks showed a similar histochemical tissue to that observed in the control group (CG), i.e., the epithelial layer moderately stained by bromophenol blue (Fig. 2D). The dermis is weakly stained by the bromophenol blue technique (dotted in Fig. 2D); for the polysaccharides, there are different reaction intensities, both for the acidic and for the neutral ones (Figs. 2E and 2F).

The cells of hair follicles strongly reacted with PAS (Figs. 2E and 2F). Increased detection of collagen fibers in the subepidermal region was observed (Fig. 2G).

Histochemical test results in treatment group I (**TGI**) are summarized in Table 2.

3.2.3. Treatment Group II (TGII)

Individuals exposed to 80% selamectin and previously infested with ticks showed an epithelial layer similar to that observed in the control group (CG), i.e., the cells were moderately stained with bromophenol blue (Fig. 3D), weakly stained by PAS, and acidic polysaccharides were not detected (Fig. 3E).

The dermis showed some areas of the connective tissue with strong PAS positive reaction (Fig. 3E), indicating the presence of neutral and acidic polysaccharides (circled in Fig. 3E). The hair follicles also strongly reacted with polysaccharides (Fig. 3F). Collagen fibers were observed (Fig. 3G).

Histochemical test results in treatment group II (**TGII**) are summarized in Table 3.

3.2.4. Treatment Group III (TGIII)

Individuals exposed to 50% selamectin and previously infested with ticks showed an epithelial layer similar to that observed in the control group (CG), i.e., cells moderately

stained by bromophenol blue (Fig. 4B), weakly stained by PAS positive, and absence of acidic polysaccharides.

The dermis in some regions of the tissue strongly reacts with PAS (neutral polysaccharide) (Fig. 4E). Collagen fibers were observed (Fig. 4D).

Histochemical test results in treatment group III (**TGIII**) are summarized in Table 4.

4. DISCUSSION

The pronounced inflammation is a process that frequently arises after minutes or hours following mechanical injury (tick attachment) to the skin of rabbits and rapidly goes through a regression causing the skin to either return to its natural state or turn into a state of chronic inflammation (Jones et al. 2000). Accordingly, in the present study the pronounced inflammation observed in the skin of rabbits was characterized by the presence of protein-rich fluids (exudates) formed outside the blood vessel in the dermis and which contain various blood cells, a set called inflammatory edema (Wilkinson et al. 1990; Jones et al. 2000; Siqueira Junior and Dantas 2000; Dos Santos et al. 2004).

In this study, skin biopsies from rabbits previously infested with *Rhipicephalus sanguineus* ticks, and subsequently exposed to different concentrations of Revolution®, were analyzed. Seventy-two hours after the procedure for attaching the ticks, the animals in the control group (**CG**) showed typical histopathological changes, which are often described in the literature (Siqueira Junior and Dantas 2000; Carvalho et al. 2010; Hebling et al., 2013), thus showing the pronounced inflammation. The hyperkeratosis found in the *stratum corneum* of the skin of rabbits might be due to the process of a cement cone formation while ticks are being attached to the rabbits. According to Balashov (1972), during engorgement, ixodid ticks would release compounds of polysaccharide and proteinaceous nature through their salivary glands. Such data were corroborated by the histochemical analyzes carried out here in. These compounds would then account for the cement cone formation in the region of the skin *stratum corneum*, leading to its hyperplasia.

Even in the control group (**CG**), the presence of epithelial hyperplasia was observed. The mammalian epidermis is regarded as a dynamic layer, since continuous movements of cell differentiation from the basal (stem cells) to the *stratum corneum* (external layer) take place there. In the basal layer that there is proliferation of keratinocytes, their differentiation when moving to the skin surface and, similarly, the corneocytes suffer peeling on the skin surface. Therefore, epidermal thickness is determined by the relative speed of these synchronous processes (Samuelson 2007; Junqueira and Carneiro 2009). If the speed of

keratinocyte mitosis is increased (hyperplasia) without any increase whatsoever in the differentiation and peeling process, then there is the formation of epithelial thickness. The thickening of the epidermis is a phenomenon known as acanthosis, a common type of change in skin diseases caused by inflammatory processes, especially by epidermal cell damage and/or a proliferative signal of the dermis in response to a pronounced inflammation (Jones et al. 2000).

In this study, after three days of infestation with *R. sanguineus* in all host groups exposed to selamectin (**TGI**, **TGII** and **TGIII**) all the couples of *R. sanguineus* ticks were found dead, but still attached to the skin. The skin of the **TGI** hosts (exposed to 100% concentration) showed the largest morphophysiological changes, whereas **TGIII** subjects (exposed to 50% concentration) showed similar changes to those in the control group (CG), i.e., the same changes in the epidermis.

The hosts in all treatment groups (**TGI**, **TGII** and **TGIII**) showed total or partial reduction of the *stratum corneum* thickness (which might promote dehydration of the skin due to exposure of the epithelium to the environment, as well as a decrease in the skin's protective barrier). According to the Secretaria de Saúde do Estado da Bahia, Brazil (2001), workers exposed to unhealthy and systemic chemicals for a certain period of time have irritant contact dermatitis (depending on the nature of the chemical, as well as the exposure time). This continuous contact with the irritant agent promotes rupture or disappearance of the skin's *stratum corneum*, leaving the dermis fully exposed. When this phenomenon occurs, there is the presence of large amounts of blood on the site. In the case of the hosts in the control group (**CG**), there was a thickening of the *stratum corneum* when they were infested with *R. sanguineus*, once the attachment of ectoparasites to the host's skin occurs by the secretion and formation of the cement cone, a process that induces skin thickness. The inflammatory process in rabbits, induced by the presence of ticks on the skin, would not cause the loss of the *stratum corneum* integrity, unlike what happened in the hosts' epidermis in the treatment groups (**TGI**, **TGII** and **TGIII**). This is most likely due to selamectin exposure, showing that higher concentration of the product, greater the morphological damage caused in the *stratum corneum* will be.

It has been reported in the literature that a wide variety of chemical compounds can potentially induce injury to several epithelial layers of many mammalian organs (Matulionis 1975; Aplemann et al. 1982; Bucley et al. 1985; Min et al. 1994; Mendes et al. 2005; Barros et al. 2006). According to Min et al. (1994), early degenerative changes induced by acute exposure to a chemical agent are easily detectable by thinning and consequent peeling of the

epithelium. In this study the thinning of the epithelium was detected, and it was also observed that such process gradually becomes stronger as the concentration of selamectin increases. Therefore, the treatment group III (**TGIII**) exposed to 50% selamectin concentration showed quite a similar epithelium to the one in the control group (**CG**); the treatment group II (**TGII**) exposed to 80% showed a slightly thickened epithelium and the lowest thickness was detected in the treatment group I (**TGI**), exposed to 100%.

Defense agents such as cathelicidin-like antimicrobial peptides of mammals constitute a component of the innate immune system. Those peptides are produced in leukocytes and have their formation generally induced in organs serving as protective barrier to the action of infectious agents, such as skin (Bals et al. 1998; Heilborn et al. 2003). Previous studies have indicated that one of the most important functions of such peptides would be to induce the process of re-epithelialization of the skin after the occurrence of lesions caused by inflammatory and infectious processes (Heilborn et al. 2003). The presence of chemicals such as selamectin might have altered the metabolism of these peptides in the skin of rabbits, so that there was no re-epithelization on the inflammation site, which led to the epithelial thinning detected in hosts exposed to different selamectin concentrations.

The pronounced formation of subepidermal edemas detected in the rabbits of the treatment groups could be related to different selamectin concentrations to which the hosts had been exposed. In an acute inflammatory process, the formation of exudate with migration of amorphous substances and a high volume of blood (such as plasma and their protein constituents) into the skin structure have been reported (Wilkinson et al. 1990; Jones et al. 2000; Siqueira Junior and Dantas 2000; Dos Santos et al. 2004). Since selamectin has a systemic action (Pfizer Central Research 2010), it is widely distributed throughout the body by the circulatory system (Krautmann et al. 2000; Novotny et al. 2000). It concentrates mainly in the regions of the glands (Hovda and Hooser 2002), which, according to Jones et al. (2000), would enable the formation of edemas. High selamectin concentrations in **TGI** and **TGII** might have induced the formation of these subepidermal edemas on the hosts' skin, which in turn induced greater migration of blood cells on the site, a fact evidenced in this study using the bromophenol blue test.

The body reaction to mechanical damage are usually acute inflammatory responses which, in the skin, make both the epidermis and the dermis start to synthesize pro-inflammatory compounds (such as TNF-α, IL-8, IL-1, LAF-1 and ICAM-1), acting in the defense process and the recovery of injured tissue (Jones et al. 2000). Although these mechanisms of cutaneous inflammation have been poorly elucidated (Nickoloff and Griffths

1990; Baker et al. 1991), it has already become clear that cells residing in the epidermis and dermis (such as epithelial cells and fibroblasts) interact, leading to the manifestation of pathological changes. Therefore, the observed disruption of collagen fibers in the dermis of the skin in all treatment groups (**TGI**, **TGII** and **TGIII**) could be a result of either the process of abundant exudate migration in the edema formation (accumulation of interstitial fluid generated by the difference in concentration gradient causing the gap between the collagen fibers) or the signaling pathways of epidermal damage. This could be explained by the fact that the thinned epidermis projection would warn leukocytes of the damage on its structure, resulting in the maintenance of edema formation in order to prevent any kind of damage to the skin structure and/or physiology.

As for strong detection of PAS-positive granulation in some regions of the dermis, as well as in the region of hair follicles in all treatment groups (**TGI**, **TGII** and **TGIII**), selamectin seems to have acted as a toxic agent. According to Hovda and Hooser (2002), the chemical agent would focus mainly on the glands present in the skin of animals. Furthermore, data available in the literature, especially toxicological tests, show that when either vertebrate and invertebrates organisms (Nath et al. 1997; Nath 2000; Albinati et al. 2009; Biagini et al. 2009) are often exposed to agents that induce toxicity in their physiology, their cells tend to store PAS-positive granules in various tissues. Generally, in case of toxicity, the processes of synthesis and storage of polysaccharides in tissues or organs are changed, since these compounds are often expected to supply and/or reduce the damage toxic agents can cause to the morphophysiology of exposed individuals (Biagini et al. 2009).

The presence of collagen fibers demonstrated by the Mallory's trichrome technique indicated the recovery process of the skin in the three treatment groups (**TGI**, **TGII** and **TGIII**) after the process of acute inflammation. After a few hours of mechanical injury suffered while *R. sangunieus* were being attached to the region of the epidermis, biochemical dermal signals to the fibroblasts were activated. It consequently activated the healing of damage through resynthesis of collagen fibers in the connective tissue that fills the dermal layer of the skin (Jones et al. 2000; Siqueira Junior and Dantas 2000), a process that might have occurred in the animals observed here.

5. CONCLUSION

The obtained results showed that selamectin acts like a toxic agent when in contact with the skin of the rabbit infested with ticks, inducing morphophysiological alterations in the acute inflammatory process in the animal's tegument. Selamectin is a chemical substance

which has a dose-dependent action, since higher the concentration, greater is the morphophysiological damage found in the skin of rabbits. When used in small doses, it can eliminate the ticks without causing considerable morphological and physiological alterations.

Acknowledgements

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Table 1. Results of histochemical tests in the tegument of rabbits infested by *Rhipicephalus sanguineus* in the control group (**CG**)

	Bromophenol Blue	PAS	Blue Alcian	Mallory's Trichrome
Stratum Corneum	**+++**	**-**	**++**	**-**
Epithelium	**++**	**+**	**-**	**-**
Dermis	**++**	**++**	**++**	**+++**
Hair Follicles	**++**	**+**	**+++**	**+**
Blood Cells	**+++**	**+**	**-**	**-**

(**+++**) strongly positive, (**++**) moderately positive, (**+**) slightly positive, (**-**) negative

Table 2. Results of histochemical tests in the tegument of rabbits infested by *Rhipicephalus sanguineus* (Acari: Ixodidae) subjected to the action of selamectin (active principle of acaricide Revolution®, Pfizer) in the treatment group I (**TGI** - Group exposed to 100% of the concentration of selamectin)

	Bromophenol Blue	PAS	Blue Alcian	Mallory's Trichrome
Stratum Corneum	**+++**	**-**	**++**	**-**
Epithelium	**++**	**+**	**-**	**-**
Dermis	**++**	**++**	**++**	**+++**
Hair Follicles	**++**	**+++**	**+++**	**+**
Blood Cells	**+++**	**+**	**-**	**-**

(**+++**) strongly positive, (**++**) moderately positive, (**+**) slightly positive, (**-**) negative

Table 3. Results of histochemical tests in the tegument of rabbits infested by *Rhipicephalus sanguineus* (Acari: Ixodidae) subjected to the action of selamectin (active principle of acaricide Revolution®, Pfizer) in the treatment group II (**TGII -** Group exposed to 80% of the concentration of selamectin)

	Bromophenol Blue	PAS	Blue Alcian	Mallory's Trichrome
Stratum Corneum	**+++**	**-**	**++**	**-**
Epithelium	**++**	**+**	**-**	**-**
Dermis	**++**	**++**	**++**	**+++**
Hair Follicles	**++**	**+++**	**+++**	**+**
Blood Cells	**+++**	**+**	**-**	**-**

(**+++**) strongly positive, (**++**) moderately positive, (**+**) slightly positive, (**-**) negative

Table 4. Results of histochemical tests in the tegument of rabbits infested by *Rhipicephalus sanguineus* (Acari: Ixodidae) subjected to the action of selamectin (active principle of acaricide Revolution®, Pfizer) in the treatment group III (**TGIII** - Group exposed to 50% of the concentration of selamectin)

	Bromophenol Blue	PAS	Blue Alcian	Mallory's Trichrome
Stratum Corneum	**+++**	**-**	**++**	**-**
Epithelium	**++**	**+**	**-**	**-**
Dermis	**++**	**+++**	**++**	**+++**
Hair Follicles	**++**	**++**	**+++**	**+**
Blood Cells	**+++**	**+**	**-**	**-**

(**+++**) strongly positive, (**++**) moderately positive, (**+**) slightly positive, (**-**) negative

Figure 1. Histological sections of the skin of rabbits infested by *Rhipicephalus sanguineus*. Control Group (CG). A. Stained by hematoxylin and eosin. B. Stained by PAS test and counterstained by blue alcian. C. General scheme. D. Stained by bromophenol blue test. E. Detail of hair follicule. F. Detail of sebaceous gland stained by hematoxylin and eosin. G. Detail of sweat gland.

bc = leukocytes; bm = basal membrane; cf = collagen fiber; de = dermis; ep = epithelium; hf = hair follicle; hy = tick hypostome; pe = pelage; sc = *stratum corneum*; sg = sebaceous gland; sw = sweat gland; dotted lines in A, B and D = epithelium. Scale bars = 20µm.

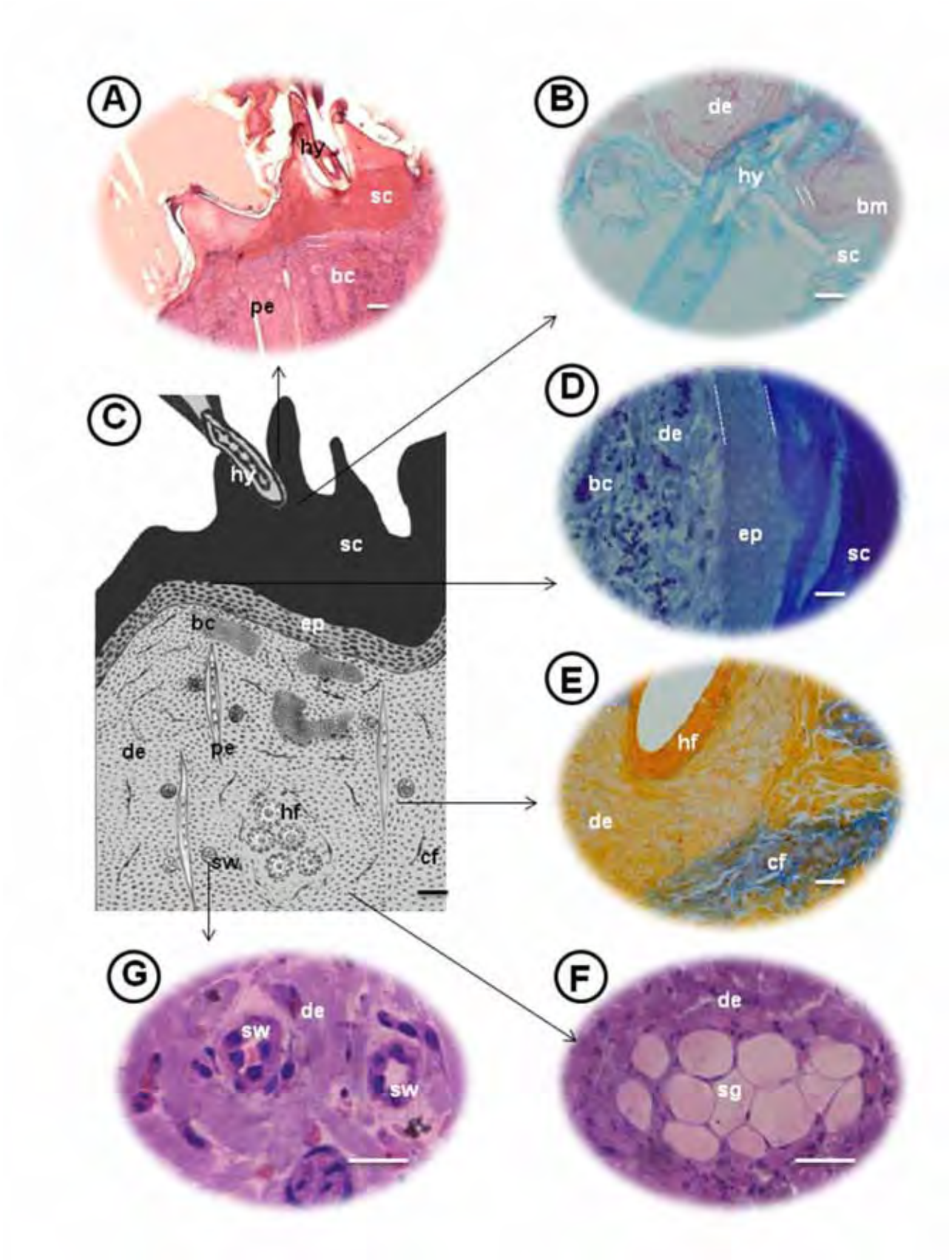
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Figure 2. Histological sections of the skin of rabbits infested by *Rhipicephalus sanguineus* subjected to the action of selamectin in concentration of 100%. Treatment Group I (TGI). A, B. Stained by hematoxylin and eosin. C. General scheme. D. Stained by bromophenol blue test. E, F. Stained by PAS test and counterstained by blue alcian. G. Stained by Mallory's trichrome technique.

bc = leukocytes; cf = collagen fiber; de = dermis; ep = epithelium; f = fibroblast; hf = hair follicle; pe = pelage; sc = *stratum corneum*; sw = sweat gland; asterisks in A, B, C and D = subepidermic edema; dotted lines in A, B and D = epithelium; dotted circle in F = acidic polysaccharides. Scale bars = 20µm.

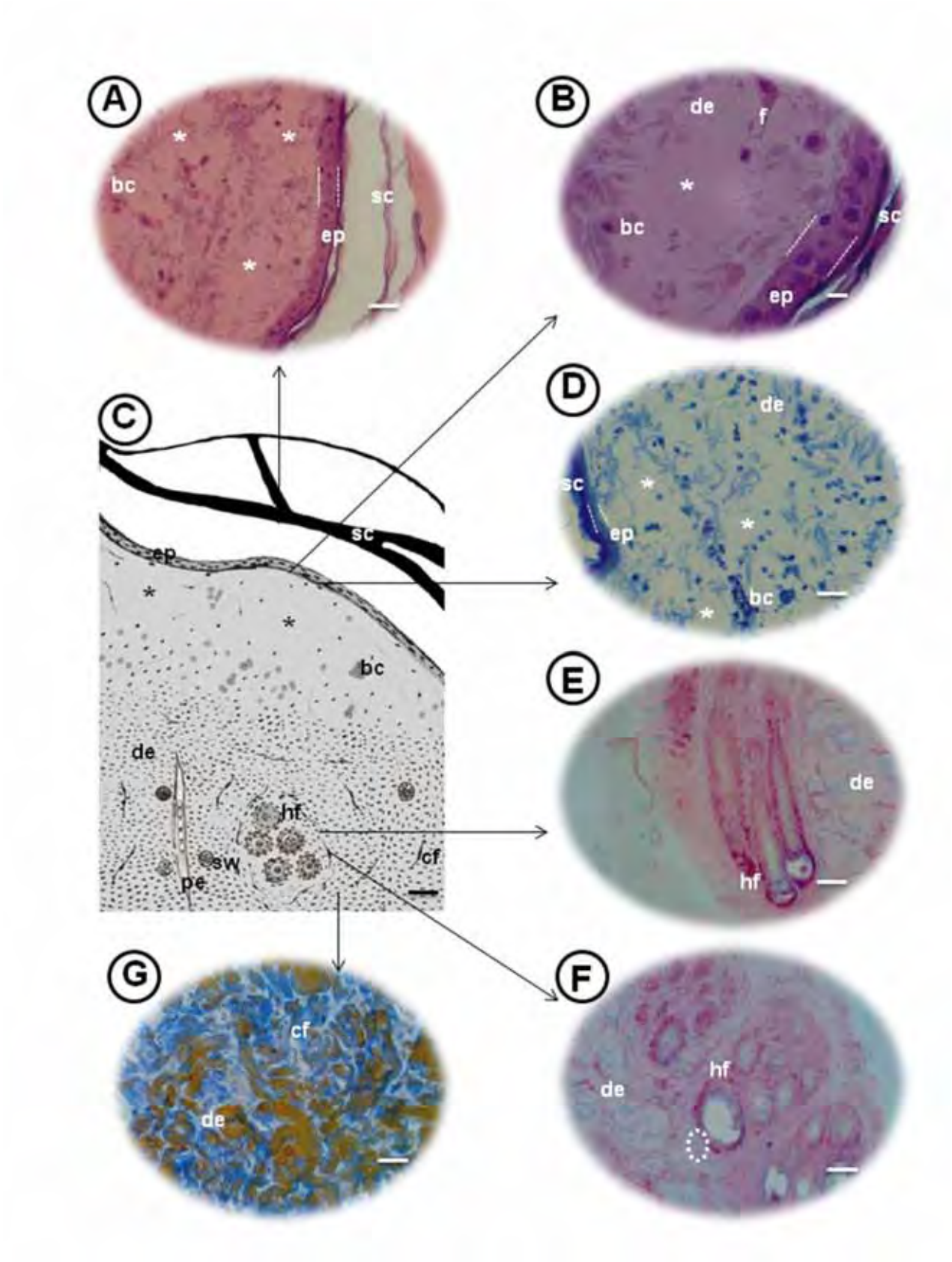
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Figure 3. Histological sections of the skin of rabbits infested by *Rhipicephalus sanguineus* subjected to the action of selamectin in concentration of 80%. Treatment Group II (TGII). A, B. Stained by hematoxylin and eosin. C. General scheme. D. Stained by bromophenol blue test. E. Stained by PAS test and counterstained by blue alcian. F. Detail of hair follicle submitted to PAS test and counterstained by blue alcian. G. Stained by Mallory's trichrome technique.

bc = leukocytes; cf = collagen fiber; de = dermis; ep = epithelium; hf = hair follicle; hy = tick hypostome; pe = pelage; sc = *stratum corneum*; sw = sweat gland; asterisks in A, C and D = subepidermic edema; dotted lines in A, B and D = epithelium; dotted circle in E and F = acidic polysaccharides. Scale bars = 20µm.

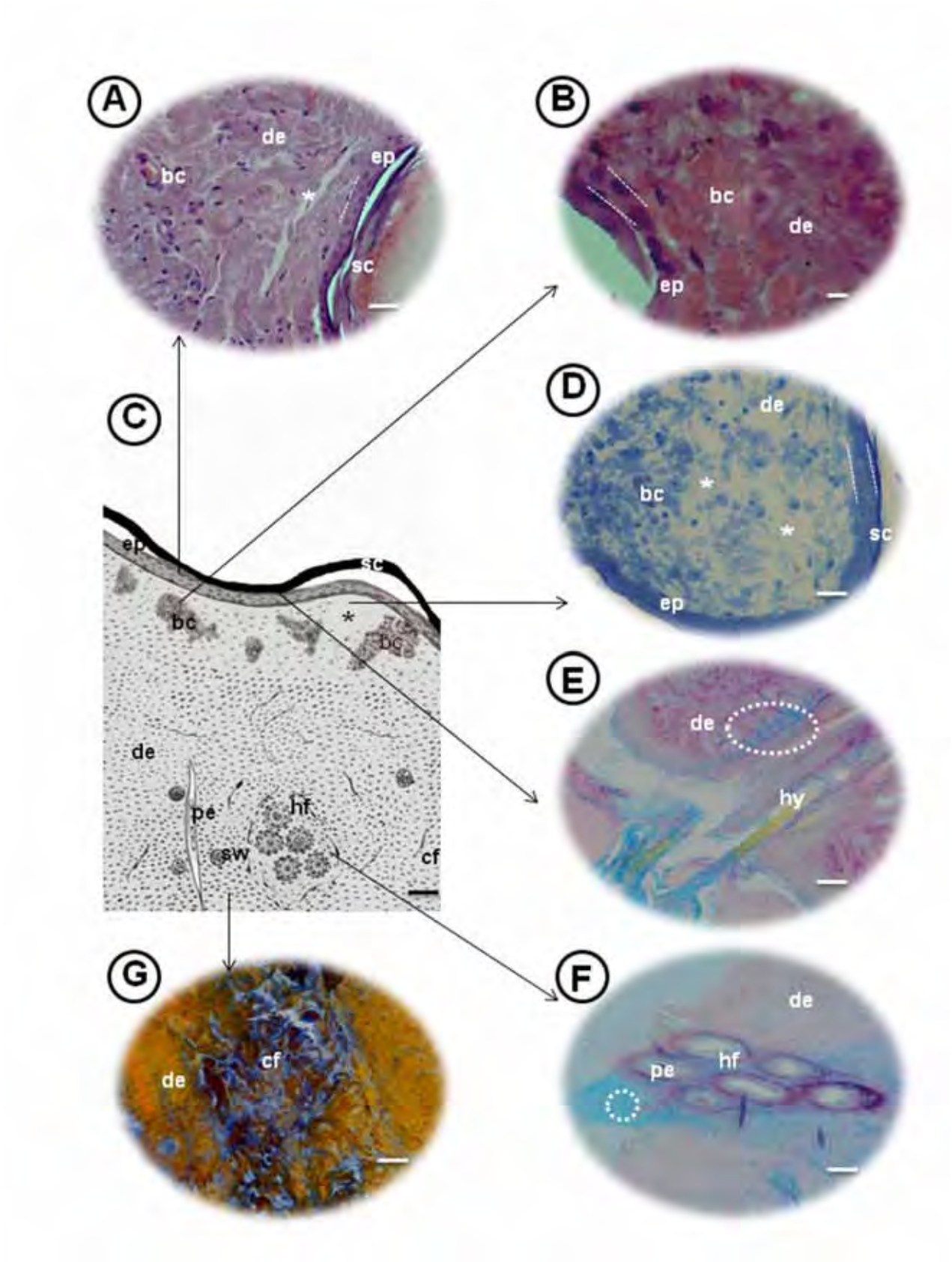
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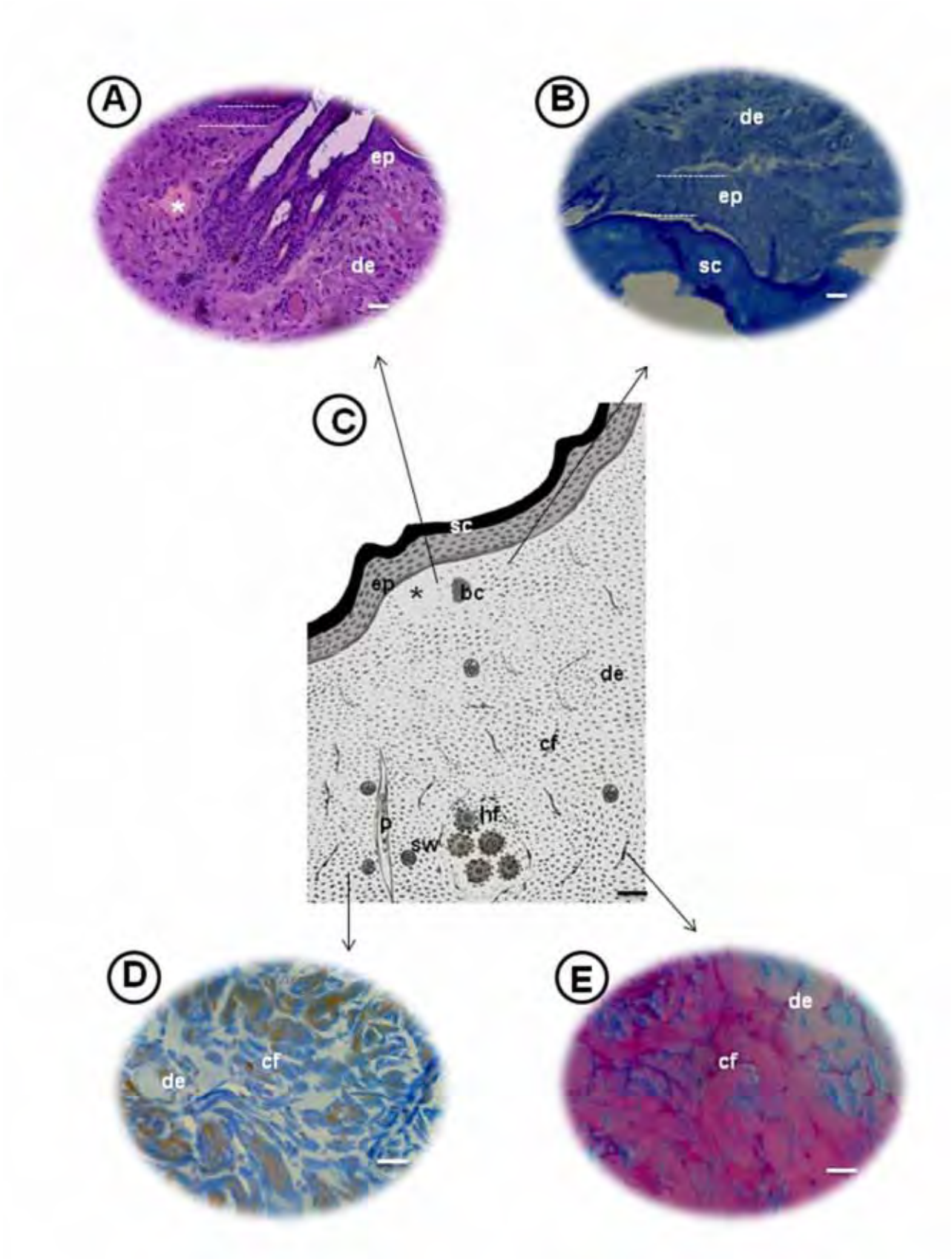
Figure 4. Histological sections of the skin of rabbits infested by *Rhipicephalus sanguineus* subjected to the action of selamectin in concentration of 50%. Treatment Group III (TGIII). A. Stained by hematoxylin and eosin. B. Stained by bromophenol blue test. C. General scheme. D. Stained by PAS test and counterstained by blue alcian. E. Stained by Mallory's trichrome technique.

bc = leukocytes; cf = collagen fiber; de = dermis; ep = epithelium; hf = hair follicle; pe = pelage; sc = *stratum corneum*; sw = sweat gland; asterisks in A and C = subepidermic edema; dotted lines in A and B = epithelium. Scale bars = 20µm.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Capítulo 2

THE OCCURRENCE OF AUTOPHAGIC CELL DEATH IN THE TEGUMENT OF RABBITS PRE-INFESTED WITH *Rhipicephalus sanguineus* AND EXPOSED TO SELAMECTIN (Active principle of acaricide Pfizer Revolution®)

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Resumo

Carrapatos da espécie *Rhipicephalus sanguineus* possuem grande importância médico-veterinária por serem vetores de diversas doenças. No intuito de minimizar a sua ação nos hospedeiros, tem-se utilizado o controle químico por meio do uso de diversos acaricidas, como a selamectina. Embora estudos prévios tenham demonstrado a sua ação tóxica em animais domésticos, nenhum estudo foi realizado na detecção de morte celular quando hospedeiros são expostos à selamectina. Por esta razão, a técnica de detecção de morte celular autofágica foi utilizada com o intuito de se demonstrar quais seriam as respostas dos tecidos da pele de coelhos pré-infestados por *R. sanguineus* e expostos a diferentes concentrações de selamectina. Os resultados obtidos mostraram que indivíduos expostos às concentrações de 100% e 80% de selamectina apresentam forte marcação à fosfatase ácida nas células do tecido conjuntivo da camada dérmica e nos folículos pilosos, enquanto que indivíduos expostos à concentração de 50% apresentam fraca marcação das células do tecido conjuntivo da derme e moderada marcação nos folículos pilosos. Ficou claro que, quando utilizada em altas concentrações (100% e 80%), a selamectina é capaz de induzir a ocorrência de processos de morte celular autofágica em larga escala. Por outro lado, a concentração de 50% provoca menores alterações morfofisiológicas na pele dos coelhos hospedeiros quando o processo de morte celular autofágico é analisado. Portanto, os dados confirmam que selamectina é um agente tóxico dose-dependente que provoca o aumento da atividade da enzima fosfatase ácida.
Palavras-chave: fosfatase ácida, hidrolase ácida, autofagia, infestação, toxicologia.

Abstract

Ticks of *Rhipicephalus sanguineus* species have great medical and veterinary importance for being a vector of various diseases. In an attempt to minimize their action on the host, people have resorted to chemical control by using various acaricides, such as selamectin. Although previous studies have demonstrated its toxic action in domestic animals, no studies focused on the detection of cell death when exposed to selamectin. For this reason, the technique for detecting autophagic cell death was used in order to demonstrate the responses of rabbits' skin tissues pre-infested with *R. sanguineus* and exposed to different concentrations of selamectin. The obtained results when exposed to 100% and 80% concentrations of selamectin showed a strong mark of acid phosphatase on the cells of the connective tissue of the dermis and hair follicles, whereas the ones exposed to the 50% concentration had a weak mark on the cells of the connective tissue of the dermis and moderate staining in hair follicles. It became clear that, when used at high concentrations (100% and 80%), selamectin is capable to induce a

large scale occurrence of the autophagic cell death process. On the other hand, the concentration of 50% causes minor morphophysiological changes in the skin of rabbit hosts when evaluated the cell death process. Therefore, the data confirms that selamectin is a powerful dose-dependent toxic agent causes increased activity of the enzyme acid phosphatase.

Key words: acid phosphatase, acid hydrolases, autophagy, infestation, toxicology.

1. INTRODUCTION

Rhipicephalus sanguineus is a cosmopolitan tick (Flechtmann, 1973) of major medical and veterinary importance, as well as one that makes the animals in which it is fixed lose large amounts of blood. It also acts as a vector of several pathogens such as protozoa, bacteria, rickettsia and viruses, bringing diseases to wild and domestic animals, even to men (Harwood and James, 1979; Dantas-Torres, 2010).

The fixation of the tick on hosts occurs when the hypostomium penetrates into their skin aided by the combined action of: a) the chelicerae that tear the tegument and b) the saliva that form the cement cone. In hosts, the tissue digestion around the hypostomium penetration canal causes the rupture of capillaries and lymphatic vessels. The tick's feeding process occurs by suction of (the host's) blood simultaneously with the inoculation of saliva produced by the salivary glands. A large amount of saliva is removed during the rapid engorgement (Balashov, 1972), which increases the host's vascular permeability, resulting in the increase of blood flow into the tick (Ribeiro, 1987).

In an attempt to minimize the action of ticks on their hosts, people have resorted to the use of chemical control (Oliveira et al., 2008; Oliveira et al., 2009; Roma et al., 2010a; Roma et al., 2010b ; Nodari et al., 2011; Pereira et al., 2011; Nodari et al., 2012; Oliveira et al., 2012; Roma et al., 2012), and several compounds such as permethrin (Roma et al. 2009), fipronil (Oliveira et al., 2011) and selamectin (Araújo et al., 2012), which have already proven their action to be effective.

Selamectin, a macrocyclic lactone of the avermectin subclass, is often used to control and efficiently remove ectoparasites of cats and dogs (Krautmann et al., 2000; Novotny et al., 2000). Although the macrocyclic lactones have a wide margin of safety, poisonings in dogs have been observed in several studies (Hopkins et al. 1990; Hadrick et al. 1995; Beal et al. 1999; Hopper et al. 2002; Snowden et al. 2006; Kenny et al. 2008). Among the main acaricides that have selamectin as their main compound is Revolution®, a product manufactured by Pfizer.

Several methodologies have been applied to detect and characterize cell death processes (Mac Gahon et al., 1995; Zakeri et al., 1995). Among the methodologies used for detection, the acid phosphatase activity --an important enzyme involved in autophagic cell death process-- stands out (Furquim et al., 2008). The acid phosphatase are acid hydrolases involved in different processes such as in bone resorption, in cell cycle control and intracellular digestion, being especially detected as part of lysosomes (Zambuzzi et al. 2005).

Despite the effectiveness and neurotoxic action of selamectin on ectoparasites (Krautmann et al., 2000; Novotny et al., 2000; Araújo et al., 2012), studies that demonstrate the occurrence of morphophysiological changes in cells and tissues of the skin of hosts exposed to this toxic (Bozzatto et al., 2013) are still scarce. The same is true about studies on the action of selamectin as the triggering agent of autophagic cell death, which is an interesting aspect to be elucidated, since selamectin is usually employed when hosts (cats and dogs) are already significantly infested, particularly with *Rhipicephalus sanguineus*. Therefore, this study uses the technique for detecting the acid phosphatase activity to provide information on the occurrence of autophagic cell death processes on the tissues of rabbits infested with *Rhipicephalus sanguineus* ticks and exposed to the action of different selamectin concentrations (Pfizer Revolution ®).

2. MATERIAL AND METHODS

2.1. Local Study

Equipment available in the facilities of the Laboratory of Histology of Biology Department - IB - UNESP - Rio Claro campus, SP, Brazil, was used. This project was submitted to and reviewed by the Ethics Commission for Animal Use in Research at UNESP - Rio Claro, SP (CEUA - IB - UNESP - CRC) and forwarded to protocol No. 9058.

2.2. Chemical Substance: Selamectin (Revolution® 12%)

Selamectin was obtained from diluting a 30mg/mL concentration of Revolution® 12% (Pfizer Animal Health), a recommended dose for animals between 2.6 and 5.0 kg (5.7 and 11 pounds). The host groups receiving treatment were exposed to different selamectin concentrations, here considered a 100% dose (the actual commercial product) and the product dilutions in distilled water at 80% and 50%.

2.3. Ticks

Rhipicephalus sanguineus semi-engorged adult couples were used. The couples were collected from a colony kept under controlled conditions (28 º C, 85% humidity and a 12-h

photoperiod) in BOD incubator, in a room of the Biotherium of Biology Department – IB – UNESP, Rio Claro campus, SP, Brazil.

2.4 Hosts

Twelve healthy rabbits (New Zealand White) of similar weight (± 3.5 kg), from the Biotério Central da UNESP, Botucatu campus, SP, Brazil, were used. They were kept in cages, appropriately fed and given *ad libitum* water, in a room of the Biotério do Departamento de Biologia – IB – UNESP - Rio Claro, SP, Brazil.

Artificial infestation of the animals, construction and fixation of the feeding chamber, as well as placement of the ticks into the chamber, followed the procedures previously described by Bechara et al. (1995).

2.5. Experimental Model

Four study groups were established according to Table 1:

Table 1. Groups of pre-infested female rabbits per *Rhipicephalus sanguineus* couples exposed to selamectin action under different concentrations

Group I (CG)	**Group II (TGI)**	**Group III (TGII)**	**Group IV (TGIII)**
3 rabbits not exposed to Revolution®	3 rabbits exposed to selamectin 100%	3 rabbits exposed to selamectin 80%	3 rabbits exposed to selamectin 50%

- Control Group (CG)

The three hosts of this group were not exposed to the product. Twenty-five unfed adult tick couples were put on them, according to the method described by Bechara et al. (1995). After three days of engorgement, the females were removed. The region of the rabbit tegument, where ticks were fixed with the hypostome, was removed (by biopsy) and placed in a suitable fixative for subsequent processing.

- Biopsy Specimen Collection of Hosts' Tissues

Skin samples were collected from the hosts (on the bite site) through punch with a 3.0 mm diameter on the spot previously anesthetized with xylocaine 2% without vasoconstrictor, a procedure performed by the veterinarian Leticia Maria Graballos Ferraz Hebling. This

technique is used to stretch the rabbit skin and insert the punch (Whyte and Perry maneuver) through rotation and vertical pressure, so that the instrument reach the desired depth (dermal skin tissue).

- Treatment Groups (TGI, TGII, TGIII)

The rabbits in the treatment groups were artificially infested with 25 fasting adult tick couples/rabbit, according to Bechara et al. (1995), and, after one day, they were exposed to the product, getting the selamectin doses proposed in Table 1. The selamectin doses were applied based on the "pour on" form (application on the body topline), as recommended on Revolution label (Pfizer Animal Health, 2012). After three days of feeding, the ticks were found dead (Araújo, personal communication). The female ticks were removed and the area where they were fixed on the hosts, together with their hypostome, was removed and biopsy material was taken for processing in the lab.

2.6. Methods

2.6.1. Detection of Acid Phosphatase Activity (Hussein et al., 1990) in the rabbits' tegument

To perform this technique, fragments of tegument resulting from the hosts' biopsy were fixed in 10% neutral buffered formalin (pH 7.4) and acetone in the proportion of 9:1 for 1 hour and 30 minutes at 4 ° C. They were subsequently washed in sodium acetate buffer (0.05 M, pH 4.8) and incubated for 45 minutes at 37°C in the medium: phosphate Naphthol AS-TR, DMSO (dimethyl sulfoxide), sodium acetate buffer (0.05M, pH 4.8), 10% MnCl2.4H2O salt and fast red violet.

For the incubation medium preparation, 3 mg of substrate naphthol AS-TR phosphate was dissolved in two drops of DMSO. Then, 10 ml of sodium acetate buffer was added. Next, 0.2 mL of 10% manganese chloride plus 6 mg fast red violet salt was added. Lastly, the final solution was vigorously mixed. The control was performed excluding the substrate (3 mg naphtol AS-TR phosphate) in the incubation medium.

The material was dehydrated in increasing concentrations of ethanol (70%, 80%, 90% and 95%), in 15 minute baths each, embedded in paraffin and sectioned. 7µm cuts were collected on glass slides and rehydrated for 1 minute in distilled water counterstained for 1 minute with Harris's hematoxylin, dried and mounted in Canada balsam for observation under a light microscope.

3. RESULTS

3.1. Control Group (CG)

The tegument of the hosts in the control group, which were only exposed to infestation by *R. sanguineus* ticks presented: a) strongly marked epithelium (Fig. 1A), b) dermal connective tissue with some weakly marked regions (Figs. 1A and 1B) and c) hair follicles moderately marked by acid phosphatase (Figs. 1A and 1B); however, the center of this follicles (medulla and hair papilla) were absent for labelling (Figs. 1A and 1B).

3.2. Treatment Group I (TGI)

The hosts' tegument exposed to selamectin 100% and infested with *R. sanguineus* ticks, presented strongly marked epithelium, connective tissue of the dermis and hair follicles (Figs. 1C and 1D).

3.3. Treatment Group II (TGII)

The hosts' tegument exposed to to selamectin 80% and infested with *R. sanguineus* ticks, showed the same labelling for the detection of the acid phosphatase activity found in treatment group I (**TGI**) (Figs. 2A and 2B).

3.4. Treatment Group III (TGIII)

The hosts' tegument exposed to to selamectin 50% and infested with *R. sanguineus* ticks, presented: a) moderately marked epithelium (Fig. 2C); b) dermal connective tissue with some weakly marked regions (Figs. 2C and 2D); and c) hair follicles moderately marked by acid phosphatase (Figs. 2C and 2D).

All results involving the detection of acid phosphatase activity are summarized in Table 2.

4. DISCUSSION

Autophagic cell death (ACD), also called type II cell death, is characterized by extensive enzymatic degradation of portions of the cytoplasm that act in acid pH, called acid hydrolases, where the acid phosphatases can be cited (Furquim et al., 2008; Kroemer and Levine, 2008). It is a mechanism that can cripple cell survival when most of the cellular structures are damaged, or when they are present in large amounts in the cytoplasm, representing a response to adverse conditions faced by the animal, such as fasting (food deprivation and consequently, nutrients), and the presence of toxins and/or pathogens (Deretic, 2006; Gozuacik and Kimchi, 2007). In the present study were observed the formation of an acute inflammatory process (data not showed) in the tegument of rabbit hosts after a 72-h fixation of *Rhipicephalus sanguineus* ticks. Accordingly, autophagic cell death was observed in all groups of the experimental model (**TGI**, **TGII** and **TGIII**), including the control group (**CG**). According to Graça et al. (2008), acid phosphatase would be a group of

enzymes that have increased activity in degenerative processes, particularly those observed during inflammation. Furthermore, these enzymes would also frequently be observed within keratinosome structures involved in skin epidermis regeneration (Matoltsy, 1966; Olson et al. 1981; Kumar et al. 2004).

Skin biopsies taken from rabbits previously infested with *R. sanguineus* ticks, subsequently exposed to different selamectin concentrations, were analyzed here. Individuals in the control group (**CG**) not exposed to the product, but infested with *R. sanguineus* ticks, showed typical acute inflammatory responses resulting from the ectoparasite attachment (thickening of the *stratum corneum* and epithelial layer of the skin, and edema formation in subepidermal layers in the dermal connective tissue) caused by mechanical damage imposed on its structure (Carvalho et al., 2010; Hebling et al., 2013), as well as a reaction of the host organism itself in an attempt to expel the foreign body (tick).

According to literature data, the cells present in the uppermost layers of the epidermal epithelial tissue, more specifically the called spiny, possess numerous electron-spherical granules measuring 50 to 100 nm in diameter (Matoltsy, 1966; Olson et al. 1981) and which, according to Kumar et al. (2004), are named keratinosomes since they contain acid phosphatase. In addition, keratinosomes are secreted into the intercellular space, spilling its contents on the plasma membrane, making this surface thicker. They are also involved in the process of desquamation of the *stratum corneum* of the skin, which occurs during the epithelial renewal (Kumar et al. 2004). In this study, it can be seen that both the individuals in the control group (**CG**) and the ones in the treatment groups I and II (**TGI** and **TGII**) showed strong marking for acid phosphatase in the epithelium, suggesting that this autophagic cell death process is already engaged in cell renewal in the epithelium of the skin. Or even that this increase in the markup can be related to the presence of greater numbers of keratinosomes, acid phosphatase positive structures involved in the process of skin regeneration, a feature commonly observed herein. In the case of the treatment group III (**TGIII**), there was weak marking for acid phosphatase, probably due to the formation of new epithelial cells during the epithelial regeneration after peeling. It was a response to an acute inflammation (Bozzatto et al. 2013) imposed in the area by laceration of the tissue, which was moderately marked by the technique in the subjects in treatment group III (**TGIII**).

The hosts in treatment groups I and II (**TGI** and **TGII**) showed strong marking for acid phosphatase in the cells of the connective tissue of the dermis, whereas the hosts in the control group (**CG**) showed weak marking in the same tissue. According to Bozzatto et al. (2013), the hosts in treatment groups I and II (**TGI** and **TGII**) showed histopathological

changes in the epidermis further intensified during the acute inflammatory response induced by the presence of high selamectin concentrations in the skin. The presence of collagen fibers, probably resynthesized after the process of acute inflammation (Bozzatto et al., 2013), could be directly influencing the occurrence of strong marking of phosphatase activity. This is due to the fact that the literature has reinforced the idea that collagen definitely influences the activity of lysosomal acid phosphatase (Oliveira et al., 2008), since higher the amount of these fibers, higher the acid phosphatase expression will be (Sicca et al. 2000; Oliveira et al. 2003; Oliveira et al., 2008). The presence of high concentrations of selamectin (100% and 80%) in **TGI** and **TGII** was added to the effect of collagen resynthesis. According to Melo et al. (2007) and Nodari et al. (2011), high concentrations of toxins such as permethrin, also used in tick control (Nodari et al., 2011) and the tityus toxin used in rat prostate cancer control (Melo et al. 2007) would greatly enhance the formation of cell death processes such as apoptosis and autophagy, thus increasing the concentrations of hydrolytic enzymes (acid phosphatase) in tissues exposed to such substances. Therefore, it can be inferred that the presence of large selamectin concentrations could be directly influencing the increase in acid phosphatase marking as observed here, since the higher the concentration of selamectin, the higher will the occurrence of autophagic cell death processes be.

Strong marking for acid phosphatase in the region of the hair follicles of rabbits was observed in subjects of treatment groups I and II (**TGI** and **TGII**). According to Cotsarelis and Pauls (1999), the hairs could suffer transient alopecia, and its follicle could have an accelerated induction of the death process during the catagenic phase (involution and fall through programmed cell death) when exposed to toxic substances and subjected to hormonal disturbances. According to some authors, in domestic animals such as cats and dogs, when exposed to different selamectin concentrations, also showed the same clinical picture (Novotny et al. 2000; Hovda and Hooser, 2002). In the present study, it was observed that high selamectin concentrations induced and accelerated the degeneration of hair follicles, thus proving intense marking for acid phosphatase in this region.

This study has shown that the hosts in treatment group III (**TGIII**) (exposed to 50% of the initial selamectin concentration) produced less intense inflammatory responses (Bozzatto et al., 2013) (resulting from the toxic effect of the product) --a fact confirmed by marking the lowest acid phosphatase--, when compared to those in treatment groups I and II (**TGI** and **TGII**). This has been calling attention to the fact that lower selamectin concentrations would already promote an effective control of *R. sanguineus* ticks, minimizing, therefore, their toxic effects on non-target host organisms.

5. CONCLUSION

When selamectin is used at 100% and 80% concentrations, it killed ectoparasites but induced the occurrence of large-scale autophagic cell death processes both in cells of the connective tissue of the dermis and in the host's hair follicles. On the other hand, at lower concentrations, it kept removing ectoparasites. Therefore, the data confirmed that selamectin is a powerful dose-dependent toxic agent, and showed that the higher the concentration used, the higher the acid phosphatase activity in the rabbit host's tegument will be.

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Table 2. Results of testing for acid phosphatase activity applied in the tegument of rabbits infested by *Rhipicephalus sanguineus* and exposed to the action of selamectin (active principle of acaricide Revolution®, Pfizer) in different concentrations.

	Control Group (**CG**)	Treatment Group I (**TGI**)	Treatment Group II (**TGII**)	Treatment Group III (**TGIII**)
Epithelium	**+++**	**+++**	**+++**	**++**
Dermis	**+**	**+++**	**+++**	**+**
Hair Follicles	**++**	**+++**	**+++**	**++**

(**+++**) strongly positive, (**++**) moderately positive, (**+**) slightly positive, (**-**) negative

Figure 1. Histological sections of the tegument of rabbits infested by *Rhipicephalus sanguineus* and subjected to the action of selamectin stained by Hussein technique for detection of acid phosphatase activity. **A.** Control Group (**CG**). **B.** Detail of hair follicles of control group (**CG**). **C.** Group exposed to selamectin at 100% - Treatment Group I (**TGI**). **D.** Detail of hair follicles of treatment group I (**TGI**).

de = dermis; ep = epithelium; hf = hair follicle; sc = *stratum corneum*; dotted circles = acidic phosphatase marking. Scale bars = 10µm.

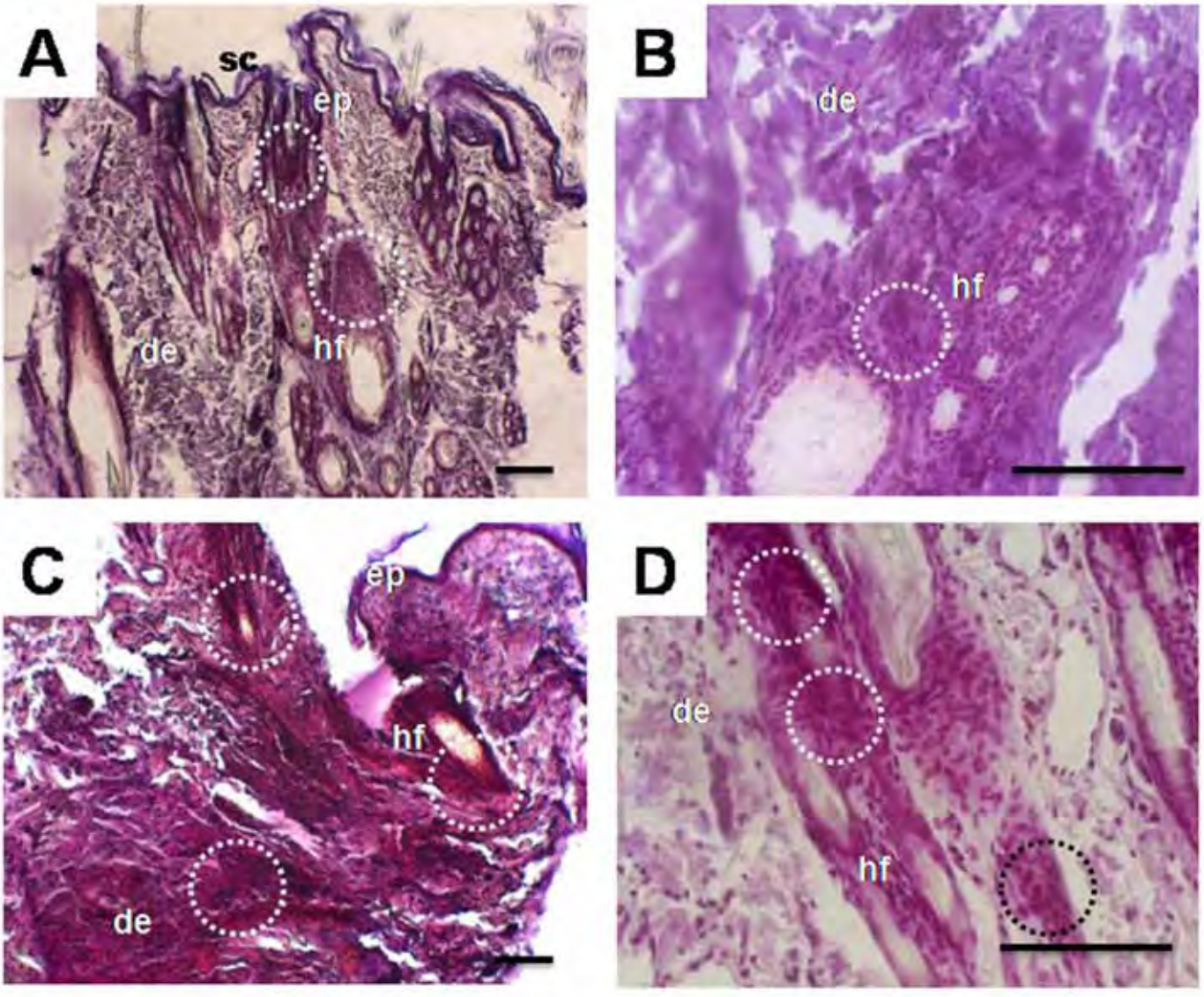
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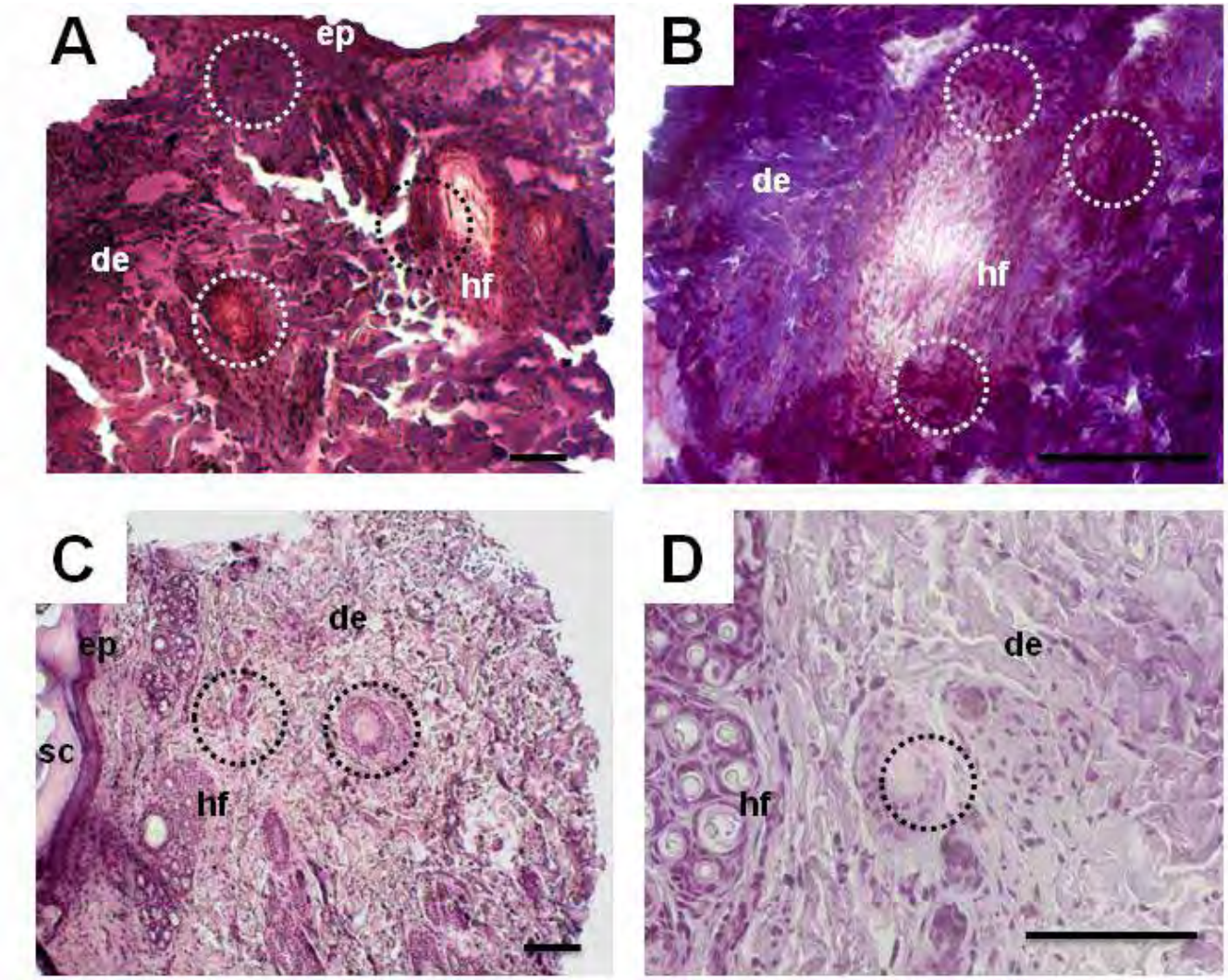
Figure 2. Histological sections of the tegument of rabbits infested by *Rhipicephalus sanguineus* and subjected to the action of selamectin stained by Hussein technique for detection of acid phosphatase activity. **A.** Group exposed to selamectin at 80% - Treatment Group II (**TGII**). **B.** Detail of hair follicles of treatment group II (**TGII**). **C.** Group exposed to selamectin at 50% - Treatment Group III (**TGIII**). **D.** Detail of hair follicles of treatment group III (**TGIII**).

de = dermis; ep = epithelium; hf = hair follicle; sc = *stratum corneum*; dotted circles = acidic phosphatase marking. Scale bars = 10µm.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Capítulo 3

MORPHOLOGICAL ALTERATIONS ON RABBITS EPIDERMIS INFESTED BY *R. sanguineus* TICKS AND EXPOSED TO SELAMECTIN (Active principle of acaricide Revolution, Pfizer): CONFOCAL MICROSCOPY

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Resumo

A espécie de carrapato *Rhipicephalus sanguineus* tem, cada vez mais, sido considerada de grande importância médico-veterinária devido ao seu potencial em transmitir doenças tanto para animais domésticos quanto para os seres humanos durante o período de fixação na pele do hospedeiro. O método mais eficaz de controle destes ectoparasitas é o uso de acaricidas químicos, dentre os quais destaca-se a selamectina. Foi objetivo deste estudo analisar, por meio de microscopia confocal de varredura a laser, a epiderme de coelhos infestados por *R. sanguineus* e expostos às concentrações de 100%, 80% e 50% de selamectina. Os resultados mostraram que coelhas expostas às concentrações de 80% e 100% (a partir do produto Revolution cuja constituição apresenta 16% de selamectina) apresentaram adelgaçamento do tecido epitelial da epiderme com consequente desorganização celular evidenciada pela forte marcação de núcleos com formas irregulares, e ainda na concentração de 100% observou-se em algumas regiões monoestratificação desta camada. Os indivíduos expostos à concentração de 50% apresentaram a epiderme com menor desorganização tecidual quando comparados aos indivíduos expostos a 80% e 100%. A selamectina, quando utilizada nas concentrações mais altas (80% e 100%) é capaz de alterar a morfologia da epiderme e em concentrações menores (50%) embora ainda capaz de eliminar os ectoparasitas ocasiona nos hospedeiros menores danos toxicológicos. A selamectina pode ser considerada um agente tóxico dose-dependente, pois quanto maior foi a sua concentração, maiores foram as alterações morfológicas na epiderme dos coelhos.

Palavras-chave: epitélio, epiderme, selamectina, acantose, toxicologia.

Abstract

The tick species *Rhipicephalus sanguineus* has had its medical-veterinary importance considered to be very increased due to its potential to transmit diseases to domestic animals and humans during attachment to the host's skin. The most effective method to control such ectoparasites is the use of chemical acaricides, among which stands out the selamectin. This study's objective was to analyze, by means of confocal laser scanning microscopy, the epidermis of rabbits infested by *R. sanguineus* and exposed to concentrations of 50%, 80% and 100% of a selamectin-based commercial acaricide (Pfizer's Revolution). The results pointed that rabbits exposed to concentrations of 80% and 100% of the product Revolution (which has 16% of selamectin in its constitution) showed epithelial tissue thinning on the epidermis with consequent cellular disorganization, evidenced by strong stainning of nuclei

with irregular shapes. At a concentration of 100%, monostratification in some regions of this layer could be observed. Individuals exposed to a 50% concentration showed lower epidermis tissue disorganization when compared to subjects exposed to selamectin 80% and 100%. The selamectin, when used in higher concentrations (80% and 100%) can alter the epidermis morphology while, in lower concentrations (50%), even though it is still able to eliminate ectoparasites, causes less toxicity damage to host. Selamectin can be considered a dose-dependent toxic agent, since higher concentrations cause more morphological changes in the epidermis of rabbits.

Key words: epithelium, epidermis, selamectin, acanthosis, toxicology.

1. Introduction

The brown dog tick belonging to the *Rhipicephalus sanguineus* has assumed an increasingly important role among tick species of medical-veterinary importance due to its great ability to transmit pathogens to host animals (DANTAS-TORRES, 2010), namely *Hapatozoon canis, Haemobartonella canis, Ehrlichia canis* and *Babesia canis*. In humans, this tick species is the vector for *Rickettsia conorii* and *R. rickettsii* (Dantas-Torres et al., 2006; Borges et al., 2007).

Balashov described in 1972 that the tick, by fixing on the host's tegument, causes various consequences such as theft by hematophagism, toxicity by inoculation of saliva comprising bioactive which would act to form the cone of cement, host immunosuppression, mechanical action by cell and tissue disruption and leather depreciation. Furthermore, the wound formed could allow the entry of microorganisms, which would cause secondary infections, such as abscesses, with consequent formation of purulent secretion (Wall and Shearer, 1997).

Several methods have been researched to achieve an effective tick control. However, the most effective is still the use of synthetic acaricides (Freitas et al., 2005). The avermectins are important anti-parasitics due to their broad-spectrum activity and efficient neurotoxic mechanism of action, which causes the paralysis of the ectoparasite by opening the chloride ion channels in the peripheral nervous system cell membranes (Novotny et al. 2000).

Selamectins, a subclass of avermectins, are macrocyclic lactones, widely used in the control of ectoparasites (Krautmann et al. 2000; Novotny et al. 2000; Araújo et al. 2012). Although the macrocyclic lactones have a wide margin of safety, poisoning in dogs and cats have been reported in some studies (Krautmann et al., 2000; Hopper et al., 2002; Snowden et

al., 2006; Kenny et al., 2008). Among the main acaricides that have selamectin as their main compound, Pfizer's Revolution® can be highlighted.

Despite the effectiveness of selamectin and its neurotoxic action on ectoparasites (Krautmann et al., 2000; Novotny et al., 2000; Araújo et al., 2012), researches that demonstrate the occurrence of morphophysiological changes in skin cells and tissues of hosts exposed to this toxic are still scarce, as well as studies dealing with the effects of selamectin on the epidermis morphology, which is an interesting aspect to be known since the epithelial layer is the first tissue contacting the stressors and/or skin pathogens. Moreover, selamectin is usually used when the hosts (dogs and cats) are already significantly infested. Thus, the present study reveals, by analyzing with confocal laser scanning microscopy, results demonstrating the occurrence of morphostructural changes in the tegument rabbit hosts infested by *R. sanguineus* ticks and exposed to different concentrations of selamectin.

2. Material and Methods

2.1. Local Study

Equipment available in the facilities of the Laboratório de Histologia do Departamento de Biologia - IB - UNESP - Rio Claro campus, SP, Brazil, was used. This project was submitted to and reviewed by the Ethics Commission for Animal Use in Research at UNESP - Rio Claro, SP (CEUA - IB - UNESP - CRC) and forwarded to protocol No. 9058.

2.2. Chemical Substance: Selamectin (Revolution® 12%)

Selamectin was obtained from diluting a 30mg/mL concentration of Revolution® 12% (Pfizer Animal Health), a recommended dose for animals between 2.6 and 5.0 kg (5.7 and 11 pounds). The host groups receiving treatment were exposed to different selamectin concentrations, here considered a 100% dose (the actual commercial product) and the product dilutions in distilled water at 80% and 50%.

2.3. Ticks

Rhipicephalus sanguineus semi-engorged adult couples were used. The couples were collected from a colony kept under controlled conditions (28 º C, 85% humidity and a 12-h photoperiod) in BOD incubator, in a room of the Biotério do Departamento de Biologia – IB – UNESP, Rio Claro campus, SP, Brazil.

2.4 Hosts

Twelve healthy rabbits (New Zealand White) of similar weight (± 3.5 kg), from the Biotério Central da UNESP, Botucatu campus, SP, Brazil, were used. They were kept in cages, appropriately fed and given *ad libitum* water, in a room of the Biotério do Departamento de Biologia – IB – UNESP - Rio Claro, SP, Brazil.

Artificial infestation of the animals, construction and fixation of the feeding chamber, as well as release of the ticks into the chamber, followed the procedures previously described by Bechara et al. (1995).

2.5. Experimental Model

Four study groups were established according to Table 1:

Table 1. Groups of pre-infested female rabbits per *Rhipicephalus sanguineus* couples exposed to selamectin action under different concentrations

Group I (CG)	**Group II (TGI)**	**Group III (TGII)**	**Group IV (TGIII)**
3 rabbits not exposed to Revolution®	3 rabbits exposed to selamectin 100%	3 rabbits exposed to selamectin 80%	3 rabbits exposed to selamectin 50%

- Control Group (CG)

The three hosts of this group were not exposed to the product. Twenty-five unfed adult tick couples were put on them, according to the method described by Bechara et al. (1995). After three days of engorgement, the females were removed. The region of the rabbit tegument, where ticks were fixed with the hypostome, was removed (by biopsy) and placed in a suitable fixer for subsequent processing.

- Biopsy Specimen Collection of Hosts' Tissues

Skin samples were collected from the hosts (on the bite site) through punch with a 3.0 mm diameter on the spot previously anesthetized with xylocaine 2% without vasoconstrictor, a procedure performed by the veterinarian Leticia Maria Ferraz Gráballos Hebling. This technique is used to stretch the rabbit skin and insert the punch (Whyte and Perry maneuver) through rotation and vertical pressure, so that the instrument reach the desired depth (dermal skin tissue).

- Treatment Groups (TGI, TGII, TGIII)

The rabbits in the treatment groups were artificially infested with 25 fasting adult tick couples/rabbit, according to Bechara et al. (1995), and, after one day, they were exposed to the product, getting the selamectin doses proposed in Table 1. The selamectin doses were applied based on the "pour on" form (application on the body topline), as recommended on Revolution label. (Pfizer Animal Health, 2012). After three days of feeding, the ticks were found dead (Araújo, personal communication). The female ticks were removed and the area where they were fixed on the hosts, together with their hypostome, was removed and biopsy material was taken for processing in the lab.

2.6. Methods

2.6.1. Confocal Laser Scanning Microscopy Of Tegument Of The Hosts

The tegument of hosts exposed to selamectin was removed and fixed in 3.4% formaldehyde. Then the material was permeated with 0.1% Triton X-100 for 20 minutes and RNAase (10 mg/ml). Thereupon, it was incubated overnight with a mixture of monoclonal anti-alpha tubulin and beta-tubulin (Sigma Aldrich) (1:100) within a moist chamber. After three washes with phosphate buffered saline (PBS), the preparations were incubated for 1h with the secondary antibody Cy5 anti-mouse (Molecular Probes) (1:100).

The material was stained with phalloidin-FITC (Sigma Aldrich) for 40 minutes and the preparations were washed in PBS in each step of each reaction. The material was mounted between slide and coverslip with anti-fading solution (Vectashield, Vector) and counterstained using 5 μL of propidium iodide (PI) (10μ/mL).

The images were obtained with a Leica TCS SP5-II Confocal Laser Scanning Microscope and analyzed with Leica TCS SP5-II and Imari software (bitplane).

3. Results

3.1. Control Group (CG)

The images obtained from the control group (CG) showed epidermal changes resulting from acute inflammatory process due to the fixation of *R. sanguineus* in the tegument, where it could be observed the occurrence of hyperplasia (thickening), confirmed by the presence of several cell layers with strong staining of nuclei of the epithelial cells (Fig. 1A).

3.2. Treatment Group I (TGI)

Individuals of the treatment group I (**TGI**), which were exposed to selamectin 100% and infested by *R. sanguineus* ticks, presented thinned epithelium with monostratification in

some regions. The layers were evidenced by strong staining of nuclei of the epithelial cells (Fig. 1B). Total tissue disorganization, paired with presence of nuclei with irregular aspect – signaling some damage – could be observed.

3.3. Treatment Group II (TGII)

Results obtained from the individuals of the treatment group II (**TGII**), exposed to to selamectin 80% and infested with *R. sanguineus* ticks, confirmed the skin thinning , phenomenon which was accompanied by total disruption of the tissue (which occurred to a lesser extent when compared to the treatment group I), including the presence of nuclei with irregular aspect, signaling damage (Fig. 1C).

3.4. Treatment Group III (TGIII)

The skin of individuals on treatment group III (**TGIII**), exposed to to selamectin 100% and infested with *R. sanguineus* ticks, also showed, by means of microscopy technique, cellular disorganization and changes in the shape of the nuclei of the epithelial layer (to a lesser extent when compared to the treatment groups I and II).

In all situations of this study the skin's autofluorescence signal was minimal.

4. Discussion

The barrier formed by the skin serves to minimize water loss from the body to the environment, reducing the absorption of chemicals, and to prevent infection from pathogens. These protective functions are mainly concentrated in the *stratum corneum*, by means of a lipid barrier and the epidermal homeostasis itself (Wickett and Visscher, 2006).

Skin biopsies taken from rabbits infested with *R. sanguineus* ticks and subsequently exposed to different concentrations of selamectin were analyzed. Individuals of the control group (**CG**) – not exposed to the product, but infested with *R. sanguineus* ticks – showed typical acute inflammatory response caused by the tick attachment to the skin (with thickening of the *stratum corneum* and the epithelial layer, paired with formation of subepidermal edemas in the dermal connective tissue) (Bozzatto et al. 2013) against the imposed mechanical injury therein (Carvalho et al., 2010; Hebling et al., 2013), as well as the reaction of the host organism itself in an attempt to remove the foreign body (tick).

The confocal laser scanning microscopy of the tegument region in the control group (**CG**), which suffered damage by ticks, confirmed the presence of acanthosis (thickening of the epithelium layer of the epidermis). The acanthosis is characterized by proliferation of the spinous cells layer and is presented evidenced by the presence of round, hypertrophied

generative cells, with dense, eosinophilic cytoplasm and persistent nuclei (Kumar et al., 2004). The latter were well evidenced by the confocal images obtained. The acanthosis is usually associated with inflammatory processes imposed on the skin, especially when there is occurrence of epidermal injury, which may occur due to mechanical shocks (here, by tissue tearing promoted by the chelicerae of ticks and subsequent fixation of the hypostome) or to biochemical signaling in dermal response to tissue regeneration (Jones et al. 2000; Kumar et al. 2004).

In the present work, the epithelial thinning phenomenon could be observed in the tegument of individuals from treatment groups I (**TGI**) and II (**TGII**). Besides presenting epithelial thinning, individuals who were exposed to 100% of the selamectin commercial dosage (**TGI**), also showed monostratification in regions of the epithelial layer. The epithelial thinning is probably a result of a cell atrophy process, which involves both decrease of the cytoplasmic and nuclear extension by loss of water from the intra to extracellular medium (Min et al. 1994; Kumar et al. 2004). This phenomenon is closely related to the process of inducing lesions to the epithelial layers by means of exposing the epithelium to xenobiotics, especially chemicals that act as toxic agents (Mendes et al. 2005; Barros et al., 2006; Trost et al., 2009). This study has shown that the highest concentration of selamectin (100% of the commercial dosage) has induced in individuals of **TGI a** sharper thinning of the epidermis, with consequent monostratification in some areas of the epithelium, responsible for the morphostructural changes herein detected. The selamectin in concentration of 80% was also able to alter the morphology of the epidermis, thus generating an epithelial thinning. However, cells appeared to be integral and the damage by the toxic action of the product seems to have a lesser extent when compared to the damage observed in the tegument of individuals in the treatment group I (**TGI**).

By analyzing images of confocal microscopy of the epidermis of individuals belonging to the treatment group III (**TGIII**), some severe changes could be observed. The results herein show that selamectin, even when used at 50% of the commercial concentration, i.e., even when used in small concentrations, is capable of controlling infestations by the *R. sanguineus* tick in the host. Morphological damage were still produced.

The present work demonstrated that the use of more advanced analysis techniques can help elucidate questions and add information about host cell and tissue damage through the action of toxic agents.

5. Conclusion

The selamectin, when used in high concentrations (80% and 100%) can alter the epidermis morphology. In lower concentrations (50%) it is still capable of eliminating ectoparasites, while causing morphological damage to hosts in a lower extent. Selamectin can be considered a dose-dependent toxic agent, since higher concentrations promoted higher morphological changes to the tegument of rabbit hosts carrying *R. sanguineus* and exposed to selamectin.

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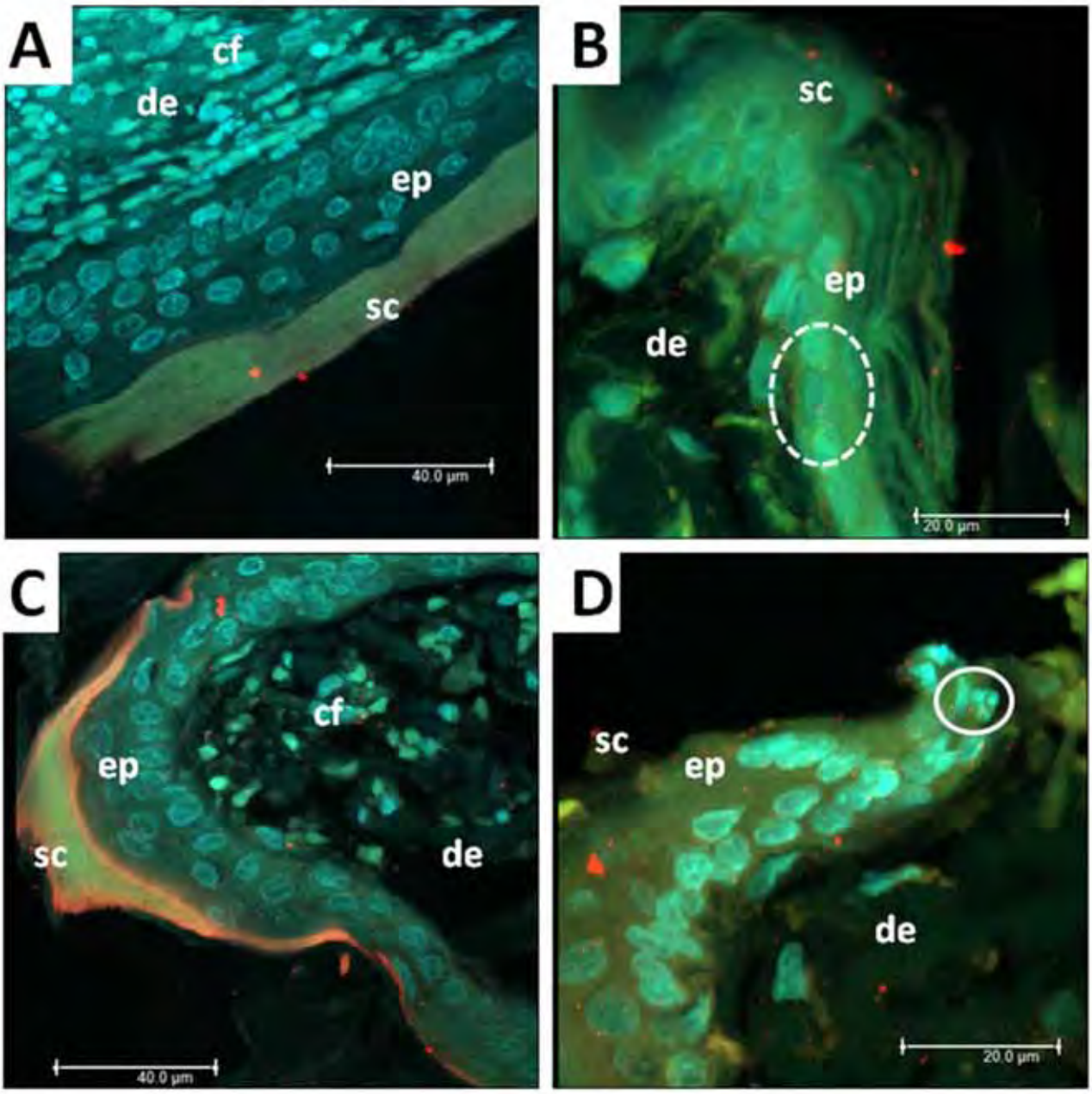
Figure 1. Images obtained by confocal laser scanning microscopy of the skin of rabbits infested with ticks *Rhipicephalus sanguineus.* **A.** Control Group (**CG**). **B.** Treatment Group I (**TGI**), subjected to selamectin at 100%. **C.** Treatment Group II (**TGII**), subjected to selamectin at 80%. **D.** Treatment Group III (**TGIII**), subjected to selamectin at 50%.

cf = collagen fiber; de = dermis; ep = epithelium of epidermis; sc = stratum corneum. Dotted circle in B = monostratification of epithelium. Continuos circles = nuclei with irregular morphology.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Discussão Geral

5. DISCUSSÃO GERAL

A pele dos mamíferos é um órgão composto de duas regiões distintas: a epiderme e a derme (SAMUELSON, 2007; JUNQUEIRA; CARNEIRO, 2009). A epiderme é dividida nas camadas: a) córnea, mais externa e em contato com o ambiente, constituída por corneócitos ricos em lipídios, b) granular, constituída principalmente por queratinócitos em processo de apoptose, c) espinhosas, com queratinócitos metabolicamente ativos sintetizando melanina e d) basal, com células-tronco essenciais à renovação do epitélio (THAKUR et al., 2008). Histologicamente, a epiderme possui externamente um epitélio estratificado pavimentoso, normalmente corneificado ou queratinizado (SAMUELSON, 2007; JUNQUEIRA; CARNEIRO, 2009). Logo abaixo à epiderme encontra-se a derme, composta por tecido conjuntivo denso não modelado que abriga vasos sanguíneos, nervos e glândulas (SAMUELSON, 2007; JUNQUEIRA; CARNEIRO, 2009). Além da epiderme e da derme, encontra-se a hipoderme, também chamada de tecido subcutâneo, rico em tecido adiposo e conjuntivo frouxo que serve para ancorar a pele aos músculos (JUNQUEIRA; CARNEIRO, 2009).

Atualmente os métodos de controle de carrapatos têm sido utilizados em larga escala principalmente em animais domésticos e de corte são via carrapaticidas químicos sintéticos, como por exemplo a selamectina. Estes produtos apresentam boa eficácia na eliminação dos ectoparasitas, entretanto podem ser desfavoráveis quanto ao seu uso, pois além de apresentar efeitos toxicológicos indesejáveis nos organismos não-alvo (hospedeiros), também podem gerar resíduos químicos no meio ambiente (POPPENGA; OEHME, 2010; OLIVEIRA et al., 2012). Exposições mínimas a substâncias químicas que tem por objetivo o controle efetivo de espécies-praga ou parasitárias podem já ser suficientes para provocar envenenamento agudo (KRAUTMANN et al., 2000; NOVOTNY et al., 2000; KENNY et al., 2008) e até gerar resíduos que afetarão diretamente a segurança pública por meio da contaminação da cadeia alimentar. O uso de carrapaticidas sintéticos pode afetar indiretamente a vida selvagem tendo como resultado a eliminação de algumas espécies que desempenham importante papel na cadeia alimentar (POPPENGA; OEHME, 2010).

A selamectina é uma substância química muito eficiente no controle de carrapatos, sendo comercializada há anos pelo laboratório Pfizer por meio do fármaco Revolution® (PFIZER SAÚDE ANIMAL, 2012), utilizada principalmente em cães e gatos. Entretanto até agora não existiam dados específicos na literatura mostrando se esta substância química seria capaz de provocar ou não alterações morfofisiológicas na reação inflamatória aguda que os carrapatos induzem na pele de seus hospedeiros, uma vez que geralmente o Revolution® é

aplicado quando cães e gatos já estão com infestações significativas. Assim sendo, o presente trabalho trouxe novos dados sobre a ação da selamectina, princípio ativo do acaricida Revolution®, uma vez que por meio de testes com diversas concentrações desta substância química na pele de coelhos infestados por carrapatos *R. sanguineus* foi possível mostrar as alterações morfológicas que as diferentes concentrações causam na pele de coelhos hospedeiros.

As alterações resultantes da ação da selamectina sobre o tegumento dos hospedeiros indicam que esta substância, quando utilizada em altas concentrações como em 100% (Grupo de Tratamento I - **GTI**) e diluída em água destilada a 80% (Grupo de Tratamento II - **GTII**), é capaz de alterar o processo inflamatório agudo da seguinte maneira: a) diminuindo parcialmente e/ou totalmente a camada córnea, b) adelgaçando o epitélio, com consequente diminuição das suas camadas celulares, c) formando edemas subepidérmicos acentuados, d) desorganizando as fibras colágenas do tecido conjuntivo da camada dérmica e e) promovendo a migração de grande quantidade de células sanguíneas para o infiltrado inflamatório, além de ter sido observado aumento da produção de polissacarídeos neutros e ácidos na região dos folículos pilosos. A microscopia confocal de varredura a laser confirmou alguns dados obtidos por meio das análises histopatológicas, mostrando o adelgaçamento no epitélio da pele dos indivíduos pertencentes a todos os grupos de tratamento. No entanto, no que diz respeito ao padrão morfológico do núcleo, ficou evidente que mesmo na concentração mais baixa (50%) a selamectina já causa mudanças morfológicas nestas estruturas.

No que diz respeito à detecção da atividade da fosfatase ácida (principal enzima envolvida no processo de morte celular autofágica), o tegumento dos indivíduos pertencentes aos grupos de tratamento I e II (**GTI** e **GTII**) apresentou epitélio, tecido conjuntivo dérmico e folículos pilosos fortemente marcados, evidenciando alta taxa de morte celular autofágica nestes tecidos, indicando que a selamectina também seria capaz de induzir a morte celular nestas estruturas.

O sistema imune inato dos mamíferos possui mecanismos de defesa não-específicos cujas respostas são indiferentes em relação ao agente estressante (JONES et al., 2010). A pele é a principal barreira deste sistema imune e constitui a primeira linha de defesa contra agentes estressantes (como por exemplo, substâncias químicas nocivas à pele) e patogênicos, sendo, portanto, o primeiro órgão a entrar em contato com estes agentes (JUNQUEIRA; CARNEIRO, 2009), e, consequentemente, o primeiro a apresentar alterações morfofisiológicas. No presente trabalho observou-se que a selamectina foi capaz de alterar o processo de resposta inflamatória aguda quando utilizada em altas concentrações (100% e

80%), reforçando a ideia de que esta substância seria um potencial agente tóxico quando em contato com a pele de coelhas hospedeiras, atuando como um agente estressante ao sistema imune inato das mesmas.

Os resultados aqui obtidos mostraram que quando a selamectina foi utilizada na concentração de 50% (Grupo de Tratamento III - **GTIII**), ocorreram respostas morfofisiológicas como: a) diminuição parcial da espessura da camada córnea, b) epitélio regularmente estratificado (muito embora com núcleos alterados, mostrados na microscopia confocal de varredura a laser) e c) formação de poucos edemas no tecido conjuntivo da camada dérmica, alterações estas que são respostas apenas à infestação por *R. sanguineus*, assemelhando-se às respostas morfisiológicas encontradas no grupo controle (**GC**). A atividade da fosfatase ácida foi semelhante àquela encontrada no tegumento dos indivíduos pertencentes ao grupo controle (**GC**), ou seja, epitélio moderadamente marcado, tecido conjuntivo dérmico com algumas regiões fracamente marcadas e folículos pilosos moderadamente marcados.

A exposição à selamectina, em altas concentrações (100% e 80%) como observado nos grupos tratamento I e II (**GTI** e **GTII**), deve ter modificado o metabolismo da pele alterando possivelmente a síntese de compostos proinflamatórios gerados tanto na epiderme quanto na derme da pele dos hospedeiros na vigência de reações inflamatórias (JONES et al., 2000; BALS et al., 1998; HEILBORN, 2003), resultando nas respostas morfofisiológicas aqui observadas. Quando utilizada em baixa concentração (50%), a selamectina também foi capaz de promover um controle efetivo dos carrapatos porém causando alterações morfofisiológicas menos intensas no tegumentos dos hospedeiros (organismos não-alvo).

Em detrimento às respostas tissulares obtidas na pele de coelhas expostas à ação da selamectina (princípio ativo do acaricida Revolution®, Pfizer) infestadas por carrapatos *R. sanguineus*, pode-se inferir que esta substância química possui ação dose-dependente, uma vez que quanto maior a concentração empregada, maiores parecem ser os danos morfofisiológicos encontrados na pele das coelhas. Quando utilizada em pequenas concentrações ela ainda é capaz de eliminar os carrapatos causando alterações morfológicas e fisiológicas menos intensas nos hospedeiros expostos.

Conclusão

6. CONCLUSÕES

Os resultados obtidos no presente estudo permitiram concluir que:

- ✓ As alterações morfofisiológicas resultantes da ação da selamectina sobre o tegumento de coelhas hospedeiras foram: a) diminuição parcial e/ou total da camada córnea, b) adelgaçamento do epitélio, c) formação de edemas subepidérmicos, d) aumento da migração de leucócitos para o infiltrado inflamatório, e) forte marcação da fosfatase ácida no epitélio, nas células da derme e nos folículos pilosos, indicando assim o seu potencial como agente tóxico para os organismos não alvo.

- ✓ Quando a selamectina é utilizada na concentração de 50% são observadas alterações morfofisiológicas menos intensas, mostrando todavia que esta dose ainda é capaz de eliminar os carrapatos com menores danos aos hospedeiros.

- ✓ A selamectina que compõe o acaricida Revolution®, Pfizer é uma substância química que possui ação dose-dependente, pois quanto maior a concentração empregada, mais intensos são os danos morfofisiológicos encontrados na pele das coelhas.

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