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MARCADORES DE ESTRESSE OXIDATIVO E ATIVIDADE DAS COLINESTERASES EM BOVINOS EXPERIMENTALMENTE INFECTADOS POR *Babesia bovis*, *Babesia bigemina* E *Anaplasma marginale*

TESE DE DOUTORADO

ROVAINA LAUREANO DOYLE

Santa Maria, RS, Brasil

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ROVAINA LAUREANO DOYLE

Tese apresentada ao Curso de Doutorado do Programa de Pós- Graduação em Medicina Veterinária, Área de Concentração em Medicina Veterinária Preventiva, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Medicina Veterinária**

Orientadora: Prof.a Dra. Cinthia Melazzo de Andrade

Santa Maria, RS, Brasil

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Universidade Federal de Santa Maria
Centro de Ciências Rurais Programa de Pós-Graduação em Medicina Veterinária
A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

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Elaborada por
Rovaina Laureano Doyle

Como requisito parcial para obtenção do grau de
Doutor em Medicina Veterinária

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RESUMO

Tese de Doutorado Programa de Pós-Graduação em Medicina Veterinária Universidade Federal de Santa Maria

MARCADORES DE ESTRESSE OXIDATIVO E ATIVIDADE DAS COLINESTERASES EM BOVINOS EXPERIMENTALMENTE INFECTADOS POR *Babesia bovis*, *Babesia bigemina* E *Anaplasma marginale*

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Data e Local da defesa: Santa Maria, 20 de fevereiro de 2015.

A Tristeza Parasitária Bovina (TPB) é uma doença que causa alta morbidade e mortalidade em bovinos suscetíveis, causada pela infecção dos protozoários *Babesia bovis* e *Babesia bigemina* e pela bactéria *Anaplasma marginale*. O objetivo deste estudo foi avaliar parâmetros de estresse oxidativo em bovinos experimentalmente infectados com *B. bovis* e *B. bigemina* e as atividades das colinesterases na infecção assintomática por *B. bigemina* assim como a interferência da esplenectomia no equilíbrio oxidativo de bovinos infectados com *A. marginale.* Para tanto, foram realizados três experimentos, sendo utilizados 24 bovinos jovens divididos em três grupos experimentais, cada um composto por oito animais sendo: no Experimento I, quatro controles e quatro infectados com cepa atenuada de *B. bovis*, onde foram observados decréscimo na contagem de hemácias e nas atividades das enzimas catalase (CAT) e superóxido dismutase (SOD) concomitantes com aumento nos níveis das substâncias reativas ao ácido tiobarbitúrico (TBARS). No experimento II, foram usados quatro bovinos controles e quatro infectados com cepa atenuada de *B. bigemina*, sendo observados decréscimo nas atividades das enzimas acetilcolinesterase (AChE), butirilcolinesterase (BChE) e CAT e aumento nos níveis de TBARS e SOD nos bovinos infectados. E, no Experimento III, quatro bovinos esplenectomizados e quatro intactos, ambos os grupos infectados com *A. marginale*, não havendo diferença entre os grupos no perfil hematológico e enzimático, apenas observada queda no hematócrito, contagem de hemácias e concentração de hemoglobina e aumento na contagem total de leucócitos devido a um aumento na contagem de linfócitos em ambos os grupos. Foram evidenciadas correlações positiva entre TBARS e a bacteremia e negativa entre NPSH e a bacteremia em ambos os grupos, porém as correlações foram maiores no grupo esplenectomizado. A partir dos resultados pode-se inferir que a infecção por *B. bovis* causa desequilíbrio oxidativo, da mesma forma que a infecção por *B. bigemina* induz a uma condição de estresse oxidativo e altera a atividade das colinesterases mesmo em animais assintomáticos e que a bacteremia por *A. marginale* influencia na peroxidação lipídica em bovinos independente da esplenectomia. Com este estudo, pode-se sugerir que marcadores de estresse oxidativo e de inflamação de baixo grau podem ser utilizados como ferramenta no auxílio do diagnóstico precoce desta enfermidade assim como servir de base para estudos referentes ao uso de antioxidantes na alimentação de bovinos para prevenir a infecção e/ou reduzir a gravidade das lesões causadas por estes parasitas.

Palavras chave: Colinesterases. Estresse oxidativo. Babesiose. Anaplasmose. Bovino.

ABSTRACT

OXIDATIVE STRESS MARKERS AND ACTIVITY CHOLINESTERASE IN EXPERIMENTALLY INFECTED CATTLE WITH *Babesia bovis*, *Babesia bigemina* AND *Anaplasma marginale*

AUTHOR: ROVAINA LAURENO DOYLE
GUIDANCE: DRA. CINTHIA ANDRADE MELAZZO
Date and defense Location: Santa Maria, February 20, 2015.

Babesiosis and anaplasmosis are part of the complex called Bovine Parasitic Sadness (TPB), a disease that causes high morbidity and mortality in susceptible cattle. It is caused by infection of *Babesia bovis* and *Babesia bigemina* protozoa and by the bacterium *Anaplasma marginale*. The objective of this study was to evaluate oxidative stress parameters in cattle experimentally infected with *B. bovis* and *B. bigemina* and the activities of cholinesterase in asymptomatic *B. bigemina* well as interference of splenectomy in the oxidative balance of cattle infected with *A. marginale*. For this, three experiments were performed, using 24 young cattle divided into three groups, each consisting of eight animals which: in the first experiment, four control and four infected with attenuated strain of *B. bovis*, which were observed decrease in erythrocytes count and activities of catalase (CAT) and superoxide dismutase (SOD) in addition to increased levels of thiobarbituric acid reactive substances (TBARS). In Experiment II, we used four cattle control and four infected with attenuated strain of *B. bigemina*, observed decrease in the activities of acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and CAT, and increased levels of TBARS and SOD in infected cattle. And, in Experiment III, four splenectomized cattle and four intact, both groups infected with *A. marginale*, with no difference between groups in the hematological and enzymatic profile, only observed drop in hematocrit, red blood cell count and hemoglobin concentration, and increased total leukocyte count due to lymphocytosis in both groups. Positive correlations were found between TBARS versus bacteremia and negative between NPSH versus bacteremia in both groups, but the correlations were higher in splenectomized group. From the results it can be inferred that infection with *B. bovis* causes oxidative balance, in the same way *B. bigemina* infection induces an oxidative stress condition and changes the atividase cholinesterase even in asymptomatic animals and bacteremia by *A. marginale* influences lipid peroxidation in independent splenectomy cattle. This study may suggest that oxidative stress and low-grade inflammation markers can be used as auxiliary tool in the early diagnosis of this disease as well as the basis for studies on the use of antioxidants in the diet of cattle to prevent infection and / or reduce the severity of injuries caused by these parasites.

Keywords: cholinesterase. Oxidative stress. Babesiosis. Anaplasmosis. Bovino.

LISTA DE ABREVIATURAS

ACh - Acetilcolina
AChE - Acetilcolinesterase
BChE - Butirilcolinesterase
CAT - Catalase
ChAT - Colina-acetiltransferase
CHT - Transportador de colina
EROs - Espécies Reativas do Oxigênio
GSH - Glutationa reduzida
GPx - Glutationa peroxidase
H_2O_2 - Peróxido de hidrogênio
HOCL - Ácido hidrocloroso
$HRO_2^{\bullet}$ - Hidroperoxil
mAChR - Receptores de acetilcolina muscarínicos
nAChR - Receptores de acetilcolina nicotínicos
MDA – Malondialdeído
NPSH – *Non-protein thiols* (Tióis não-protéicos)
O_2 - Oxigênio
$O_2^{\bullet-}$ - Ânion superóxido
$OH^{\bullet}$- Radical hidroxila
PCR - Reação em Cadeia da Polimerase
RBC – *Red blood cells* (Hemácias)
$RO_2^{\bullet}$ - Peroxil
SH - Grupo sulfidrila
SNC - Sistema Nervoso Central
SOD - Superóxido dismutase
TBARS - Substâncias reativas ao ácido tiobarbitúrico
TPB – Tristeza Parasitária Bovina
VAChT - Transportador de acetilcolina vesicular

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APRESENTAÇÃO

Os resultados dos experimentos que fazem parte desta tese estão apresentados sob a forma de artigos científicos, os quais se encontram nos itens ARTIGOS. Essa tese de Doutorado está organizada seguindo a estrutura e apresentação de monografias, dissertações e teses (MDT) 2014. O item CONSIDERAÇÕES FINAIS, encontrado no final desta tese, apresenta as interpretações discutidas sob um ponto de vista que buscou estabelecer uma conectividade entre os objetivos e resultados obtidos nos artigos contidos neste trabalho.

As REFERÊNCIAS BIBLIOGRÁFICAS se referem somente às citações que aparecem nos itens INTRODUÇÃO e CONSIDERAÇÕES FINAIS desta tese.

Os artigos estão estruturados de acordo com as normas das revistas científicas para as quais foram submetidos:

Artigo I: *Comparative Clinical Pathology*

Artigo II: *Research in Veterinary Science*

Artigo III: a submeter.

Os experimentos *in vivo* descritos nesta tese foram desenvolvidos no Instituto de Pesquisas Veterinárias Desidério Finamor, assim como as análises moleculares qualitativas de *Babesia bigemina* e *Babesia bovis.* As análises hematológicas foram feitas no Laboratório de Análises Clínicas Veterinárias da UFSM, sob orientação das Professoras Cinthia Melazzo de Andrade e Sonia Terezinha dos Anjos Lopes, as análises enzimáticas foram feitas nos Laboratórios de Enzimologia e Toxicologia (EnziTox) e de Bioquímica e Estresse Oxidativo (BioOx) da UFSM. As análises moleculares quantitativas foram feitas no laboratório de Hemoplasmas da Universidade de Purdue em West Lafayette, Indiana, EUA, sob orientação da Prof. Joanne Belle Messick

1. REVISÃO DE LITERATURA

1.1.Tristeza Parasitária Bovina

A Babesiose e a anaplasmose compõe o complexo denominado de Tristeza Parasitária Bovina (TPB). Este complexo é causado por protozoários do gênero *Babesia*, transmitidos por carrapatos da família *Ixodidae* e bactérias do gênero *Anaplasma*, transmitidos por carrapatos e insetos hematófagos (RYMASZEWSKA e GRENDA, 2008). No Brasil são reconhecidos como agentes etiológicos da Babesiose, *Babesia bigemina* e *Babesia bovis* e, da Anaplasmose, *A. marginale.* Estes micro-organismos podem ser transmitidos pelo carrapato monoxênico *Rhipicephalus* (*Boophilus) microplus* (BERENGUER, 2006; RIET-CORREA et al., 2001), além de insetos hematófagos, fômites contaminados e de forma iatrogênica estarem envolvidos na transmissão do *A. marginale* (GUGLIELMONE, 1995; KOCAN et al., 2010; MARTINS e CORRÊA, 1995; MONTEIRO, 2010). A TPB se manifesta com febre, anemia, icterícia, prostração, anorexia, edema na face e isolamento que determinaram a denominação do termo Tristeza, sendo responsáveis por altas taxas de mortalidade em populações suscetíveis. Os sinais clínicos variam dependendo da espécie e da virulência da cepa do parasito, do inóculo e da sensibilidade do hospedeiro (raça, idade, individual) (KESSLER et al., 1998; MARTINS e CORRÊA, 1995; MONTEIRO, 2010; RODRIGUES et al., 2005; WEISS e WARDROP, 2011). No Brasil, as perdas econômicas diretas e indiretas causadas pela TPB, foram estimados em mais de R$ 500 milhões (GRISI et al., 2002). No Rio Grande do Sul, as perdas causadas pela mortalidade de bovinos vitimados pela TPB foram estimadas em R$ 3,7 milhões (ALMEIDA et al., 2006). Fonseca e Braga (1924) relataram que:

> "No nosso país a piroplasmose grassou (...) causando graves devastações e estorvando o melhoramento de nossos rebanhos."

1.1.1 *Babesia* spp.

Babésias são protozoários do filo apicomplexa que podem infectar os eritrócitos de vários animais domésticos e silvestres. São descritas parasitando bovinos, as espécies *B. bigemina* (SMITH e KILBORN, 1893) e *B. bovis* (BABES, 1888), descritas no Brasil e consideradas de maior importância econômica (KESSLER et al., 1992; MONTEIRO, 2010);

além de *Babesia divergens*, *Babesia major* e *Babesia ovata*. Também são descritas em búfalos: *B. bigemina*, *B. bovis* e *Babesia orientalis*; em pequenos ruminantes: *Babesia motasi*, *Babesia ovis*, *Babesia taylori* e *Babesia foliata*; em suínos: *Babesia trautmanni* e *Babesia perroncitoi*; em equinos: *Babesia equi* (atualmente *Theileria equi*) e *Babesia cabali* e em cães: *Babesia canis canis*, *B. canis vogeli*, *B. canis rossi* e *B. gibsoni* (UILENBERG, 2006; WEISS e WARDROP, 2011). Em humanos, são descritas as espécies *Babesia microti*, *Babesia divergens*, *Babesia duncani* e *abesia. venatorum* (KJEMTRUP e CONRAD, 2000).

No Brasil, o único vetor descrito é o carrapato monoxênico dos bovinos *Rhipicephalus (Boophilus) microplus*, sendo a transmissão transovariana unicamente descrita para babésias (KESSLER et al., 1992; MONTEIRO, 2010). Na epidemiologia mundial, a babesiose tem sua ocorrência dependente da presença do vetor (KESSLER et al., 1998)

Na corrente circulatória do hospedeiro mamífero, babésias se multiplicam assexuadamente por esquizogonia ou fissão binária, sendo observadas no interior dos eritrócitos sob as formas redonda, ovalada, alongada, amebóide, trofozoítos e em pares piriformes, geralmente bigeminados, os merozoítos (GARDINER et al., 1989; MONTEIRO, 2010) (Figura 1).

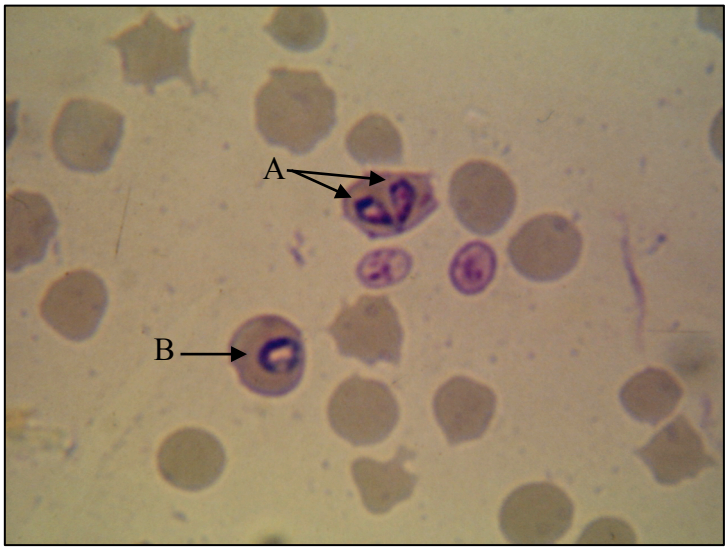

Figura 1: Esfregaço de sangue de bovino experimentalmente infectado por *Babesia bigemina*. A. Merozoítos bigeminados no interior do eritrócito; B. Trofozoíto no interior do eritrócito. (Panóptico Rápido – 1000x). Foto: João Ricardo Martins.

O ciclo biológico de babésias inicia quando o carrapato, ao se alimentar, inocula os esporozoítos (Figura 2-1) que penetram nas hemácias do hospedeiro, transformam-se em trofozoítos (Figura 2-2) e se dividem assexuadamente por divisão binária (merogonia) (Figura 2-3) formando os merozoítos (Figura 2-4).

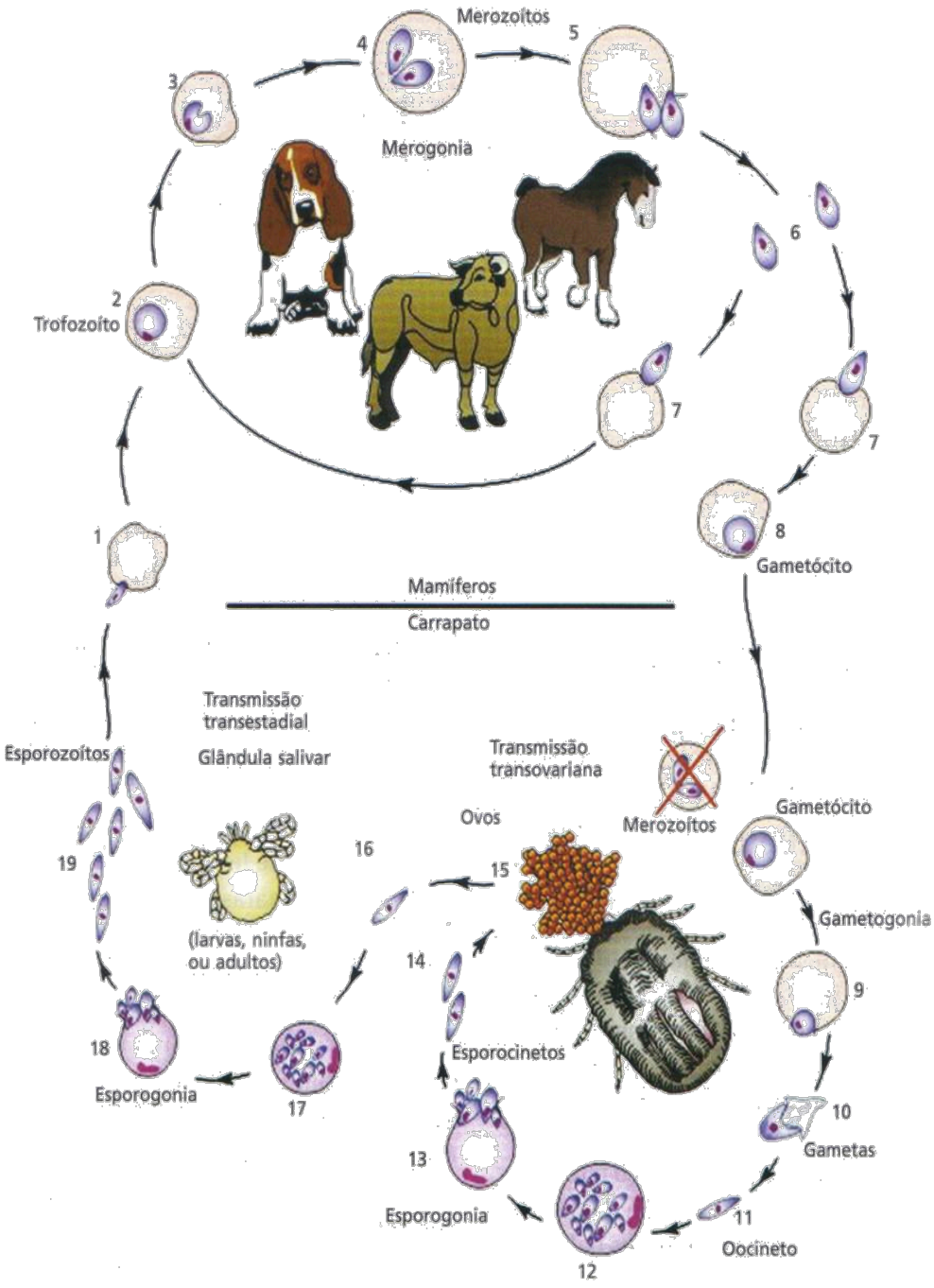

Figura 2: Ciclo biológico de *Babesia spp*. Ilustrado por Luis Augusto Salgado. Fonte: MONTEIRO, 2010.

A hemácia se rompe (Figura 2-5) e os merozoítos são liberados (Figura 2-6) penetrando em novas hemácias e reiniciando a multiplicação (Figura 2-7). Uma pequena porcentagem dos merozoítos não se divide e se transforma em gametócitos esféricos (Figura 2-8) que, ao serem ingeridos pelo carrapato vetor, iniciarão o ciclo sexuado.

Os merozoítos são destruídos no intestino do carrapato, enquanto os gametócitos se diferenciam em gametas masculinos e femininos (Figura 2-9) que se reproduzem por gametogonia (Figura 2-10) formando o oocineto (gameta com motilidade) (Figura 2-11) o qual penetra nas células do intestino do carrapato e se multiplica por divisão binária ou esporogonia (Figura 2-12) originando esporocinetos (Figura 2-13), também chamados de vermículos (organismos claviformes alongados que podem ser detectados na análise microscópica da hemolinfa). Os vermículos circulam pela hemolinfa do carrapato (Figura 2-14), infectando vários órgãos, inclusive os ovários, podendo infectar parte dos seus ovos (Figura 2-15), resultando na formação dos esporozoítos (corpos piriformes) (Figura 2-16) nas células das glândulas salivares das larvas do carrapato, onde se multiplicam por esporogonia (Figura 2-17) e formam os esporozoítos (Figura 2-18) que serão inoculados nos bovinos pelos carrapatos (Figura 2-19) (GARDINER et al., 1989; KESSLER et al., 1998; MONTEIRO, 2010).

Em 1893, Smith e Kilborn denominaram de *Pyrosoma bigeminum* o agente parasita causador de hemólise e transmitido por carrapatos ixodídeos, estudando a, então chamada, Febre do Texas (FONSECA e BRAGA, 1924; HUTYRA et al., 1953; UILENBERG, 2006).

Babesia bigemina é conhecida como grande babésia bovina, medindo de 3 a 5 micrômetros de comprimento por dois de largura. Este hemoparasita começa a ser inoculado no estágio ninfal do carrapato, ou seja, em torno de oito dias após a fixação das larvas do carrapato, permanecendo em incubação por 6 a 14 dias antes de aparecerem os primeiros sinais clínicos dependendo da taxa de inoculação e da sensibilidade do hospedeiro. As manifestações clinicas geralmente aparecem quando a parasitemia excede 1%, podendo ultrapassar 40% de eritrócitos infectados na fase aguda (KESSLER et al., 1998; MAHONEY e MIRRE, 1979; MAHONEY et al., 1973; SOULSBY e MÖNNIG, 1968).

Os sinais clínicos incluem febre, anorexia, prostração, evoluindo para a hemoglobinúria e anemia (SOULSBY e MÖNNIG, 1968). Na fase hemolítica aguda, a anemia é normocítica, mais tarde se torna macrocítica, sendo evidenciados policromasia, anisocitose, pontilhado basofílico, poiquilocitose, metarrubrícitos, reticulócitose e leucopenia (FONSECA e BRAGA, 1924; GARDINER et al., 1989; RODRIGUES et al.,

2005; WEISS e WARDROP, 2011). A anemia hemolítica é causada pela remoção e destruição dos eritrócitos infectados, causadas pela lesão física da multiplicação do parasita, devido ao aumento da fagocitose dos eritrócitos pelos macrófagos ativados, pela produção de anticorpos anti-eritrócitos e pelo aumento da permeabilidade da membrana eritrocitária (ALKHALIL et al., 2007; GOES et al., 2007; WRIGHT, 1979). A oxidação dos eritrócitos inclui lesão na membrana, formação de metahemoglobina, fragilidade osmótica e destruição celular (HARVEY, 2001).

Babesia bovis foi a primeira babésia a ser descrita, identificada por Babés, em 1888 na Romênia, sendo denominada de *Haematococcus bovis* e a doença de Hemoglobinúria bacteriana. Em 1893, Stacovici renomeou o agente etiológico como *Babesia bovis*. Em 1901, Francisco Fajardo identificou os piroplasmas no Brasil, em bovinos recém importados (FONSECA e BRAGA, 1924; UILENBERG, 2006).

Babesia bovis é considerada uma pequena babésia, medindo menos de três micrômetros de comprimento podendo ser inoculada nos bovinos por larvas do carrapato, já no primeiro dia do parasitismo, com período pré-patente de 6 a 12 dias. *B. bovis* é considerada a mais patogênica devido a alterações neurológicas e vasculares como aumento da permeabilidade vascular, estase circulatória e choque desencadeados pela ativação da calicreína plasmática induzida pela multiplicação do parasita nos eritrócitos (MARTINS e CORRÊA, 1995; MONTEIRO, 2010). Além disso, os eritrócitos infectados por *B. bovis* tornam-se rígidos e apresentam alterações na superfície da membrana e formação de protusões que favorecem a adesão das hemácias parasitadas ao endotélio capilar principalmente do cérebro (GOHIL et al., 2010; MONTEIRO, 2010) (Figura 3), desencadeando o quadro clínico conhecido como Babesiose cerebral ou nervosa, em que são observados sinais de incoordenação motora, andar cambaleante, opistótono, cegueira, andar em círculos, pressão da cabeça contra objetos, movimentos de pedalagem, ataxia, agressividade e coma. Outros sinais clínicos observados incluem hemoglobinúria, anorexia, febre, taquicardia, taquipnéia e queda na produção de leite (MARTINS e CORRÊA, 1995; UILENBERG, 2006). A infecção por *B. bovis* geralmente apresenta baixa parasitemia, em torno de 0,04 a 0,2% (MAHONEY et al., 1973).

Após a invasão da célula hospedeira, alguns parasitas intracelulares permanecem dentro do vacúolo parasitóforo que pode ou não fundir-se com lisossomas, no caso de *B. bovis*, abandona o vacúolo e se estabelece no compartimento citosólico, o que representa um passo crítico no seu processo de escape (ANDREWS e WEBSTER, 1991).

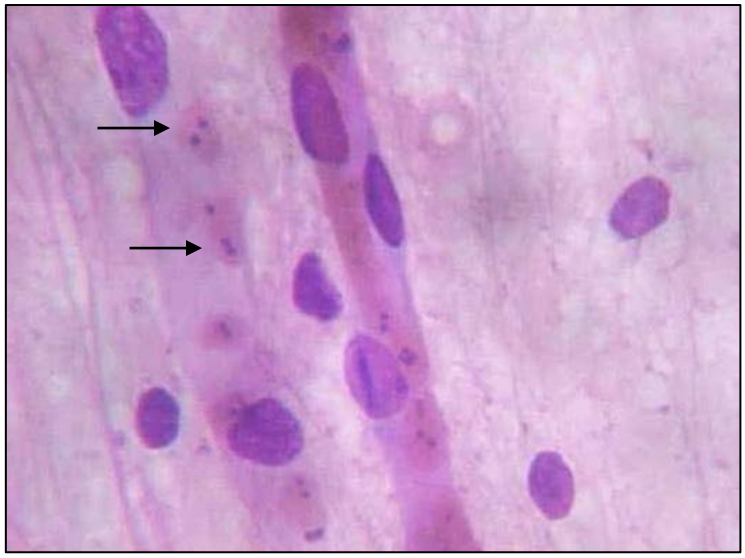

Figura 3: Imprint de cérebro de bovino com babesiose cerebral. Setas indicam hemácias parasitadas por *Babesia bovis* nos capilares cerebrais (Panóptico Rápido – 1000x). Foto: João Ricardo Martins.

1.1.2. *Anaplasma* spp.

A primeira descrição de *Anaplasma* foi feita por Smith e Kilborne em 1893, na América do Norte, ainda considerados uma forma de desenvolvimento de babésias. Theiler em 1910, na África os denominou 'pontos marginais' e diferenciou da Febre do Texas (Babesiose). No Brasil foi descrita primeiramente por Carini em 1910, sendo *A. marginale* a espécie representativa, uma vez que as demais espécies deste gênero, *A. centrale* e *A. ovis,* têm pouca importância patogênica (HUTYRA et al., 1953; MARTINS e CORRÊA, 1995).

São bactérias gram negativas, atualmente diferenciadas das rickettsias pois estas possuem vários gens para síntese de lipopolissacarídeos, os quais não foram identificados no gênero *Anaplasma* (BRAYTON et al., 2005).

A. marginale possui várias proteínas na superfície da membrana externa, denominadas 'Major Surface Proteins' (msp) que podem ser usadas para identificação molecular da bactéria assim como podem atuar como antígenos para indução da resposta imune do hospedeiro (BRAYTON et al., 2005; CORONA et al., 2005; LÖHR et al., 2002). Algumas dessas proteínas sofrem variações antigênicas permitindo a evasão da resposta

imune do hospedeiro, sendo responsáveis pela infecção permanente de *A. marginale* nos bovinos (BRAYTON et al., 2005; DE LA FUENTE et al., 2001).

Além dos bovinos, *A. marginale*, também pode infectar ovelhas, cabras, búfalos, cervídeos, gnus e antílopes, mas sem produzir a doença clinica. O único animal silvestre que pode apresentar manifestações clinicas de anaplasmose é a girafa (KUTTLER e JOHNSON, 1986).

A anaplasmose pode ser transmitida biologicamente por carrapatos e mecanicamente por dípteros hematófagos, porém não tão eficaz quanto os primeiros (BOWMAN, 2010; DE LA FUENTE et al., 2001).

No Brasil, a transmissão biológica é feita pelo carrapato *R. (B.) microplus*, porém não são descritas transmissão transovariana e transestadial (KESSLER et al., 1998; RIET-CORREA et al., 2001), sendo os carrapatos machos responsáveis pela transmissão (DE LA FUENTE et al., 2001). Nos Estados Unidos, após a erradicação do carrapato vetor *R. (B.) microplus*, a transmissão biológica passou a ser mantida pelo carrapato *Dermacentor andersoni* porém com menor capacidade vetorial, pois as larvas e ninfas parasitam pequenos ruminantes, apenas os carrapatos adultos se alimentam em bovinos (FUTSE et al., 2003).

A maior capacidade vetorial dos carrapatos é conferida pelas moléculas anti-hemostáticas, antiinflamatórias e imunomediadas presentes na saliva destes, pois estes compostos alteram a fisiologia no local da picada no hospedeiro, facilitando a entrada de patógenos inoculados junto com a saliva durante a hematofagia (VALENZUELA, 2004).

Em áreas endêmicas onde há alta população destes vetores, os animais podem ser infectados nos primeiros dias de vida, enquanto ainda estão protegidos pela imunidade passiva, sendo que o parasita aparece entre 50 a 74 dias de idade, geralmente com parasitemia baixa a moderada (ERIKS et al., 1989). O pico da parasitemia fica em torno de 7% e ocorre de 1-4 semanas após o aparecimento dos primeiros eritrócitos infectados em esfregaços sanguíneos (KESSLER et al., 1998; RISTIC, 1981).

Os sinais clínicos incluem anemia hemolítica progressiva, febre, perda de peso, queda na produção de leite, abortos e morte (JONES et al., 1968; KESSLER et al., 1998; RISTIC, 1981). No início da infecção, há remoção somente das hemácias parasitadas que apresentam alterações celulares. Com a evolução da patogenia, aparecem os auto-anticorpos que aderem aos eritrócitos infectados e não infectados, aumentando a fagocitose das hemácias pelos macrófagos, principalmente no baço (RISTIC, 1981).

A patogênese da anemia é principalmente imunomediada por anticorpos que lesionam a membrana dos eritrócitos infectados ou não, causando hemólise extravascular (STOCKHAM e SCOTT, 2011). Esta intensa destruição das hemácias aumenta a produção biliar, causando distensão na vesícula pela presença de bile espessa e grumosa (‘mal da bile’). A insuficiência hepática permite a passagem de sais e ácidos à circulação, que podem determinar uma toxemia. As alterações da bile favorecem as disfunções digestivas que terão como consequências hepatoesplenomegalia, icterícia, coprostase ou diarreia, dentre outros distúrbios (MASSARD et al., 1998). Além da anemia severa, pode ocorrer reticulocitose, policromasia e pontilhado basofílico acompanhados de marcada hiperbilirrubinemia e bilirrubinúria (STOCKHAM e SCOTT, 2011).

Em esfregaços sanguíneos corados, normalmente são visualizadas, de uma a duas inclusões basofílicas de 0,55 a 0,85µm (também chamadas de corpo elementar) nas bordas das hemácias de animais doentes (Figura 4), cada corpo elementar pode conter uma a oito subunidades, reconhecidas como as formas infectantes, inoculadas pelos carrapatos durante o repasto sanguíneo (CORONA et al., 2005).

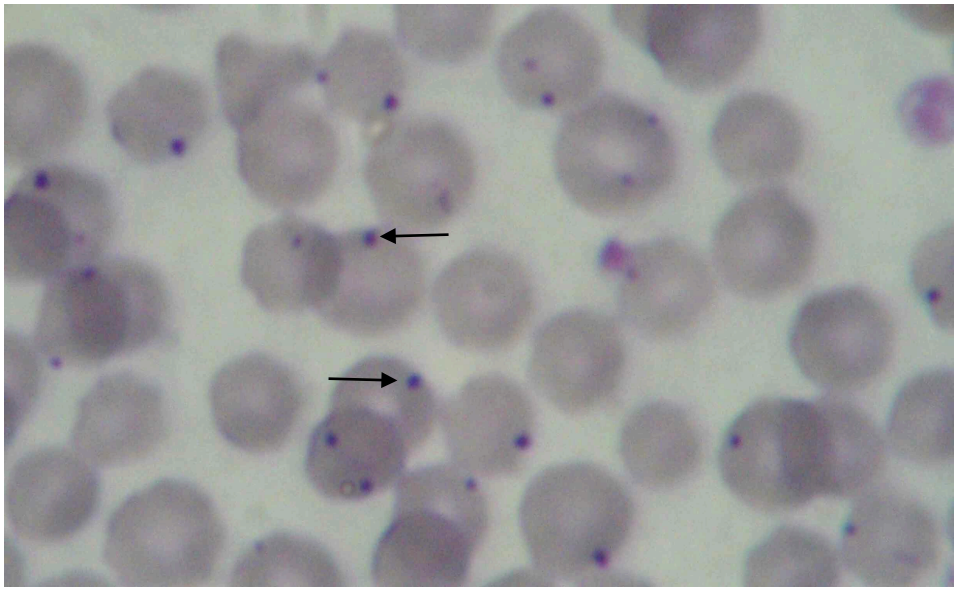

Figura 4: Esfregaço de sangue de bovino infectado por *Anaplasma marginale.* Setas evidenciam as inclusões nas bordas das hemácias (Panóptico rápido – 1000x). Foto: arquivo pessoal.

Estes corpúsculos iniciais se aderem às hemácias do novo hospedeiro e penetram por invaginação da membrana citoplasmática, ocorrendo o “embolsamento” do parasito com

posterior formação do vacúolo parasitóforo. A multiplicação do corpúsculo inicial é feita por divisão binária e então, forma-se o corpo elementar que abandona o eritrócito por mecanismos não líticos (CORONA et al., 2005; MARTINS e CORRÊA, 1995; MASSARD et al., 1998; RIBEIRO e REIS, 1981).

Na fase aguda da infecção por *A. marginale* a parasitemia pode atingir 50% dos eritrócitos. Os animais que sobrevivem à fase aguda da anaplasmose tornam-se portadores crônicos com bacteremia cíclica indetectável em esfregaço sanguíneo (HUTYRA et al., 1953).

1.1.3. Achados de necropsia da babesiose

Durante a necropsia de bovinos com babesiose, podem ser observadas mucosas anêmicas, baço e fígado escuros, aumentados e congestos, linfonodos intumescidos e escuros, vesícula biliar distendida, com bile escura, densa e grumosa e hidropericárdio. Em bovinos infectados por *B. bovis*, também pode ser observada congestão do córtex cerebelar e cerebral e coloração róseo-cereja da massa cinzenta, enquanto na anaplasmose, podem ser detectadas mucosas anêmicas e ictéricas, baço aumentado, fígado amarelado e aumentado e vesícula biliar obstruída (MENDES et al., 2009; RODRIGUES et al., 2005).

1.1.4. Epidemiologia da TPB

B. bigemina tem a ocorrência dependente da presença do seu vetor e está distribuída na África, Ásia, Austrália, Américas Central e do Sul e sul da Europa, enquanto *B. bovis* tem ocorrência semelhante, porém menos generalizada na África (BOCK et al., 2004). Em 1906, os Estados Unidos lançaram uma campanha para erradicação do carrapato *R. (B.) microplus*, vetor da babesiose no continente norte-americano, o que eliminou virtualmente a doença em 1940 deste país (BOWMAN, 2010). Enquanto *A. marginale* ocorre em zonas tropicais e subtropicais, independente da presença de carrapatos (KOCAN et al., 2010; PALMER et al., 1999).

A incidência e a gravidade dos sinais são maiores em animais adultos, uma vez que os animais jovens (até oito meses) geralmente desenvolvem a doença subclínica devido à resistência inata (MADRUGA et al., 2001; WEISS e WARDROP, 2011).

Praticamente todo o estado do Rio Grande do Sul tem a característica de instabilidade enzoótica para a TPB, além de possuir condições climáticas que determinam períodos mais ou menos longos sem a infestação por carrapatos. Como consequência ocorre uma queda no nível de anticorpos contra os agentes da TPB e também a predominância da criação de raças européias, mais sensíveis ao carrapato e, portanto, expostas a maiores inócuos, sendo frequentes os surtos com elevadas morbidade e mortalidade (ARTILES et al., 1995; RIET-CORREA et al., 2001).

1.1.5. Diagnóstico diferencial

A TPB pode ser confundida com leptospirose, clostridiose, raiva, haemoncose, tripanossomose, enfermidades causadas pela ingestão de plantas tóxicas como *Ateleia glazioviana* (timbó), *Cestrum laevigatum* (coreana), *Cassia occidentalis* (sin. *Senna occidentalis* – fedegoso), *Pteridium aquilinum* (samambaia), *Brachiaria radicans* (Tanner grass) e *Senecio* spp. (Maria-mole), além de desequilíbrios alimentares como intoxicação por cobre e deficiência de fósforo (KESSLER et al., 1992; ARTILES et al., 1995; KARAM et al., 2002).

1.1.6. Tratamento

O tratamento de bovinos com TPB é feito com drogas de efeito babesicida (derivados da diamidina), anaplasmicida (tetraciclinas) ou de dupla ação (imidocarb ou associações de diamidina com oxitetraciclina) (BOCK et al., 2004).

1.1.7. Controle e Profilaxia

O controle deve ser feito através de medidas de manejo adequadas à epidemiologia dos agentes da TPB na região. Nas áreas de instabilidade enzoótica deve-se manter uma população mínima de carrapatos, capaz de manter o rebanho imune (KESSLER et al., 1998; RIET-CORREA et al., 2001).

Uma alternativa bastante utilizada, principalmente quando há a introdução de animais com baixa imunidade em áreas endêmicas é a quimioprofilaxia em que são administradas

subdosagens de derivados do imidocarb, as quais permitirão ao animal adquirir a infecção sem sinais clínicos ou com sinais brandos. (KUTTLER e JOHNSON, 1986).

A imunidade persiste enquanto o animal permanece portador da infecção latente, no caso de *B. bigemina*, este período pode durar até 2 anos, entretanto, se houver uma eliminação da infecção, seja por auto-esterilização, tratamento efetivo ou remoção total do carrapato vetor, esta imunidade é perdida em 12 a 14 meses (SOULSBY e MÖNNIG, 1968), enquanto a imunidade adquirida contra *B. bovis* pode durar quatro anos (MAHONEY e ROSS, 1972). No caso de infecção por *A. marginale*, que na fase aguda apresenta alta parasitemia, após a remissão, um baixo nível de infecção indetectável ao esfregaço sanguíneo persiste por vários anos, mantendo a imunidade dos bovinos (HUTYRA et al., 1953; KOCAN et al., 2010; MARTINS e CORRÊA, 1995).

1.2. Sistema colinérgico

O sistema colinérgico é um dos mais importantes caminhos modulatórios do Sistema Nervoso Central (SNC), sendo fundamental em várias funções vitais relacionadas com o aprendizado, a memória, a organização cortical do movimento, bem como a regulação do fluxo sanguíneo cerebral, o que faz deste sistema um importante alvo de pesquisa (MESULAM et al., 2002).

Os principais componentes do sistema colinérgico são a acetilcolina (ACh), a colina-acetiltransferase (ChAT); o transportador de colina (CHT); o transportador de acetilcolina vesicular (VAChT); os receptores de acetilcolina muscarínicos (mAChR) e nicotínicos (nAChR) e as colinesterases: acetilcolinesterase (AChE) e butirilcolinesterase (BChE) (MESULAM et al., 2002) as quais serão o foco deste trabalho, devido à escassez de estudos sobre a atividade das colinesterases na anaplasmose e babesiose experimentais em bovinos.

1.2.1 Acetilcolina

A acetilcolina (ACh) foi a primeira molécula identificada como neurotransmissor, passando a ser amplamente estudada nas sinapses e junções neuroefetoras colinérgicas dos SNC e periférico (PRADO et al., 2002). A ACh também regula os níveis e as atividades da serotonina, dopamina e de outros neuropeptídeos e, portanto, modula tanto neurotransmissão quanto a resposta imune (DAS, 2007).

1.2.2 Sinapse colinérgica

A ACh é sintetizada no citosol do neurônio pela enzima ChAT a partir de uma molécula de colina e acetil-coenzima A ou acetil-CoA. Posteriormente, este neurotransmissor é armazenado dentro de vesículas sinápticas pelo VAChT. Com a chegada do potencial de ação a ACh é liberada na fenda sináptica e exerce seus efeitos mediados pela ativação de receptores nicotínicos e muscarínicos (KUTTY, 1980; SILVA, 1998). A ACh que permanece na fenda sináptica é hidrolisada por colinesterases específicas (MESULAM et al., 2002; RANG e DALE, 2007). Existem dois tipos de colinesterases: a acetilcolinesterase (AChE; E.C 3.1.1.7) ou colinesterase verdadeira que hidrolisa preferencialmente ésteres com grupamento acetil (como a ACh) e a butirilcolinesterase (BChE; E.C. 3.1.1.8) ou pseudocolinesterase que hidrolisa outros ésteres como a butirilcolina (BRADY et al., 2005).

1.2.3 Acetilcolinesterase

A AChE possui um papel regulatório na neurotransmissão colinérgica, uma vez que é responsável pela hidrólise rápida da ACh, encontrada nos neurônios colinérgicos, nas proximidades das sinapses colinérgicas e em concentrações elevadas na junção neuromuscular (MASSOULIÉ et al., 1993; SOREQ e SEIDMAN, 2001). A AChE está amplamente distribuída no SNC e também é encontrada em tecidos não neurais como eritrócitos, plaquetas e linfócitos de mamíferos (ÇOKUĞRAŞ, 2003; SILVA, 1998). Nos linfócitos acredita-se que esta enzima represente um importante papel na regulação de funções imunes (KAWASHIMA e FUJII, 2000) e também é encontrada em células progenitoras do sangue, onde pode efetuar atividade relacionada à hematopoiese (SOREQ e SEIDMAN, 2001).

Em adição, a AChE também tem potentes efeitos sobre a adesão celular, na neurogênese, na sinaptogênese e atividade hematopoiética pela presença desta enzima em células progenitoras do sangue (SILMAN e SUSSMAN, 2005; SOREQ e SEIDMAN, 2001). No sangue a atividade da AChE é considerada um bom marcador periférico de alterações no SNC por apresentar propriedades funcionais semelhantes às das AChE encontrada na fenda sináptica (THIERMANN et al., 2005). Por isso um aumento ou uma inibição desta enzima pode resultar em consequências importantes tanto no cérebro quanto em outros órgãos

(SILVA et al., 2006).

A AChE existe nas formas globular e assimétrica. A forma globular é composta por monômeros (G1), dímeros (G2) e tetrâmeros (G4) da subunidade catalítica. A forma G1 é citosólica e a G4 é ligada a membrana, sendo esta última a mais encontrada no tecido nervoso (DAS et al., 2001). No sangue a AChE é encontrada tanto nos eritrócitos quanto no plasma, onde predominam as formas G2 e G4 respectivamente. Já a forma assimétrica consiste de um (A4), dois (A8) e três (A12), tetrâmeros catalíticos ligados covalentemente a uma subunidade estrutural colagênica chamada Q (CoIQ). Essas formas estão associadas com a Lâmina basal e são abundantes na junção neuro muscular (ALDUNATE et al., 2004).

1.2.4. Butirilcolinestease

A BChE é uma enzima sérica produzida no fígado, sendo principalmente encontrada no plasma, rins, intestino, massa branca do cérebro, pulmão e em algumas glândulas endócrinas e exócrinas (KUTTY, 1980; MESULAM et al., 2002b). A BChE não é eficiente em hidrolisar ACh em baixas concentrações, mas pode substituir a AChE na degradação da ACh quando a mesma estiver inibida, demonstrando que ela atua quando há uma maior disponibilidade de neurotransmissor (LI et al., 2006).

1.2.5 Colinesterase e processo inflamatório

Uma nova propriedade da AChE e BChE foi identificada como marcadores inflamatórios de baixo grau (DAS, 2007). Vários estudos têm demonstrado que a ACh, o principal neurotransmissor vago tem importantes ações antiinflamatórias. No entanto, a ACh, bem como outros ésteres de colina, são rapidamente hidrolisados pela AChE e BChE (MESULAM et al., 2002). Um aumento nas atividades das enzimas AChE e BChE poderia levar à diminuição nos níveis de ACh, reduzindo seus efeitos antiinflamatórios, devido à ausência do controle de feedback negativo exercido pela ACh (RAO et al., 2007). Dessa forma, considerando o efeito inflamatório supressor da ACh, é aceitável que as atividades das enzimas AChE e BChE sejam reguladoras intrínsecas da inflamação (ANGLISTER et al., 2008; DAS, 2007).

1.3. Estresse oxidativo

1.3.1. Espécies Reativas do Oxigênio (ERO)

Os radicais livres são moléculas que contém um ou mais elétrons desemparelhados nas órbitas externas, o que os torna muito instáveis, lábeis e quimicamente muito reativos (CHIHUAILAF et al., 2002; HALLIWELL e GUTTERIDGE, 2007). As ERO incluem radicais livres como o ânion superóxido ($O_2^{\bullet -}$), peroxil ($RO_2^{\bullet}$), hidroperoxil ($HRO_2^{\bullet}$) e o radical hidroxila ($OH^{\bullet}$) (Molina et al., 2003), este último é considerado o mais reativo por combinar-se rapidamente com metais, podendo causar danos como mutação ou inativação do DNA celular, além de iniciar a oxidação dos ácidos graxos poliinsaturados das membranas celulares (lipoperoxidação) (FERREIRA e MATSUBARA, 1997; HALLIWELL et al., 2000). As espécies não radicalares, apesar de não possuírem elétrons desemparelhados, são muito instáveis como, por exemplo, o peróxido de hidrogênio (H_2O_2) e o ácido hidrocloroso (HOCL) (TURKO e MURAD, 2002).

O H_2O_2 é um metabólito do oxigênio extremamente deletério, pois participa da reação que produz o radical hidroxila ($OH^{\bullet}$) (reação de Fenton). O H_2O_2 tem vida longa, é capaz de atravessar camadas lipídicas, pode reagir com a membrana do eritrócito e com proteínas ligadas ao ferro, o que o torna altamente tóxico para as células. Esta toxicidade aumenta em presença de ferro, que é o metal pesado mais abundante no organismo e capaz de catalisar as reações de oxidação de biomoléculas (FERREIRA e MATSUBARA, 1997; HALLIWELL et al., 2000).

O estresse oxidativo é definido como o excesso de formação e/ou remoção insuficiente de moléculas reativas, tais como: espécies reativas de oxigênio (ERO) e espécies reativas de nitrogênio (ERN) (BRITO et al., 2007; SIES, 1994).

Os danos oxidativos causados nas biomoléculas do organismo pelas ERO incluem a peroxidação lipídica, a oxidação protéica e o dano no DNA celular (Figura 5) (CHIHUAILAF et al., 2002; HALLIWELL e GUTTERIDGE, 2007; YU, 1994).

Entretanto, a produção de ERO é de extrema importância no combate aos agentes infecciosos, uma vez que estas lesões oxidativas são nocivas às estruturas celulares dos parasitas, auxiliando o sistema imune da defesa contra a invasão do organismo (MARR e MULLER, 1995).

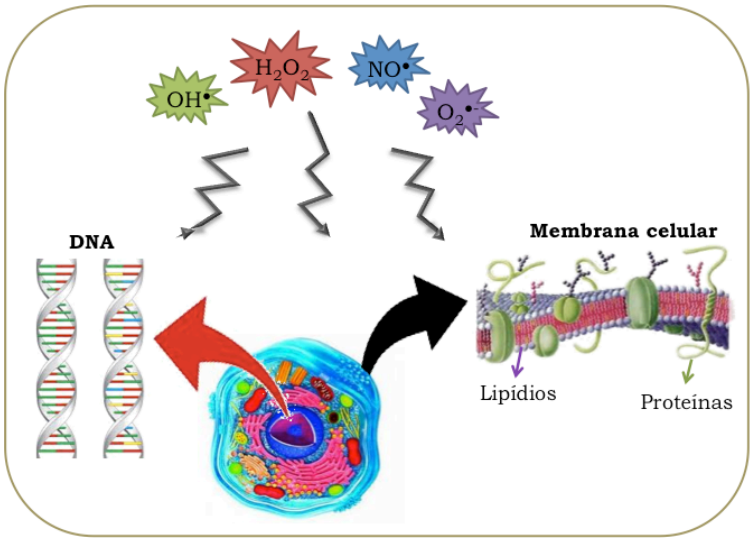

Figura 5: Esquema ilustrativo do alvo das espécies reativas de oxigênio (ERO) nas biomoléculas do organismo (lipídios, proteínas e DNA). Ilustração da autora.

1.3.2. Peroxidação lipídica

A peroxidação lipídica inicia quando as ERO atacam ligações duplas ou triplas de ácidos graxos poliinsaturados alterando sua conformação química inicial, sendo que estas reações após iniciarem se auto-perpetuam. Como conseqüências podem ser observadas alterações na integridade estrutural, perda da fluidez e aumento da permeabilidade a íons das células (CHIHUAILAF et al., 2002).

Outro problema desta reação é a formação de Fe^{3+}, que pode reagir com peróxidos lipídicos formando os radicais peroxilas e Fe^{2+}, em um ciclo autossustentável. A hemólise dos eritrócitos ocorre devido à peroxidação lipídica da membrana juntamente com a liberação do Fe^{2+} (HALLIWELL e GUTTERIDGE, 2007; KOURY e DONANGELO, 2003). O processo de lipoperoxidação forma produtos como gases de hidrocarbonetos e aldeídos, como o malondialdeído (MDA) (HALLIWELL e GUTTERIDGE, 2007).

O metabolismo lipídico pode estar envolvido na invasão das células hospedeiras, na formação de vacúolo parasitóforo pelas babésias o que pode levar a deformações na membrana celular. A membrana do vacúolo parasitóforo é formada imediatamente após a invasão da célula hospedeira através da atividade dos lipídios organelares (roptrias) (MARR e MULLER, 1995). A peroxidação lipídica tecidual é avaliada através dos níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS), que é provavelmente o método mais

comumente aplicado para sua mensuração (ESTERBAUER, 1993). Um aumento na peroxidação lipídica provoca dano tecidual e está envolvido em diversas condições patológicas (HALLIWELL e CHIRICO, 1993).

A gravidade da infecção por *B. bigemina* está diretamente relacionada à carga parasitária, com a peroxidação lipídica da membrana do eritrócito, juntamente com a formação de metahemoglobina, fatores que agravam a fragilidade osmótica e a hemólise intravascular, desempenhando papel fundamental na patogênese da anemia causada por esta espécie (SALEH, 2009). Além disso, estas alterações oxidativas aumentam a eliminação destas células pelo baço (MORITA et al., 1996).

Alguns estudos têm demonstrado aumento dos níveis de MDA sérico em cães com babesiose (CHAUDHURI et al., 2008; CRNOGAJ et al., 2010). Também foi demonstrada elevação dos níveis de MDA nos eritrócitos de bovinos com *B. bovis* (COMMINS et al., 1988), *B. bigemina* (SALEH, 2009) e na theileriose bovina (ASRI REZAEI E DALIR-NAGHADEH, 2006; SHIONO et al., 2001). Deger et al. (2009) demonstraram um aumento da peroxidação lipídica e uma redução da atividade de glutationa reduzida (GSH) em equinos naturalmente infectados por *T. equi*.

1.3.3. Mecanismos antioxidantes

O organismo possui um sistema de proteção antioxidante, enzimático e não enzimático, que tem a importante função de inibir os efeitos deletérios das EROs através do equilíbrio entre agentes pró-oxidantes e antioxidantes (CHIHUAILAF et al., 2002; HALLIWELL e GUTTERIDGE, 2007).

Em relação ao sistema antioxidante enzimático, pode-se destacar a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx), que constituem a primeira linha de defesa endógena de neutralização das ERO. Através destas enzimas, as células tentam manter baixas as quantidades do radical superóxido e de peróxidos de hidrogênio, evitando assim, a formação do radical hidroxila (HALLIWELL e GUTTERIDGE, 2007). A SOD é uma metaloenzima que participa do processo de detoxificação dos radicais livres, ela é específica na remoção do radical superóxido, catalisando a sua dismutação a peróxido de hidrogênio, através da reação que transforma dois ânions de radical superóxido ($O_2^{\bullet}$) em um peróxido de hidrogênio menos reativo que o anterior, como demonstrado na Figura 6.

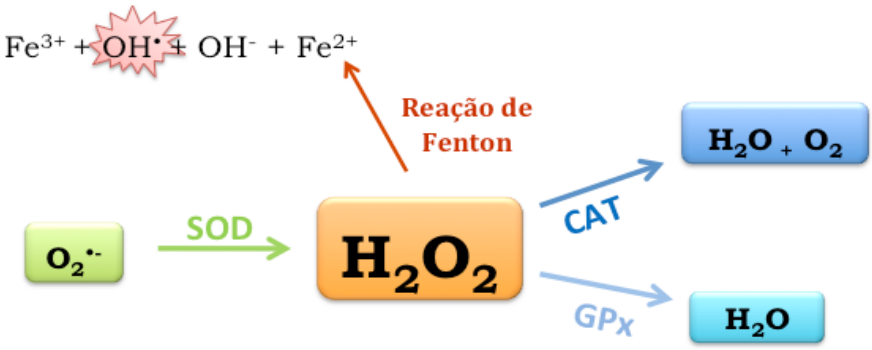

Figura 6: Esquema ilustrativo das reações catalisadas pela Catalase (CAT), Superóxido Dismutase (SOD) e Glutationa Peroxidase (GPx) e a Reação de Fenton. Ilustração da autora.

O peróxido de hidrogênio formado é degradado pela ação da CAT ou da GPx, resultando em H_2O e O_2. O H_2O_2 é capaz de atravessar a membrana nuclear e induzir danos na molécula de DNA por meio de reações enzimáticas (ANDERSON, 1996) (Figura 6).

Estudos demonstraram que a resposta hemolítica está associada ao estresse oxidativo, evidenciado pela formação de H_2O_2 e ERO (COHEN e HOCHSTEIN, 1964). Em adição, Chaudhuri et al. (2008), relataram um aumento na atividade das enzimas antioxidante (SOD e CAT) nos eritrócitos de cães naturalmente infectados por *B. gibsoni*. Já, Wallace e Dimopoullos (1965) demonstraram que os eritrócitos de bovinos infectados por *A. marginale* continham uma maior atividade de CAT que os bovinos sadios. Este achados sugerem um mecanismo compensatório do organismo em aumentar a atividade das enzimas antioxidantes na tentativa de neutralizar a formação das ERO formadas durante o processo inflamatório na babesiose e na anaplasmose.

Dentre os antioxidantes não enzimáticos podem-se destacar as vitaminas C e E além dos compostos orgânicos contendo grupos sulfidrila (SH) denominados tióis não protéicos (*Non protein thiols* – NPSH). A vitamina C apresenta propriedades antioxidantes protegendo várias moléculas contra o dano causado pelas ERO (HALLIWELL et al., 2000). Além de sua ação direta contra radicais livres, o ácido ascórbico ou vitamina C afeta indiretamente o balanço entre antioxidantes e oxidantes, já que promove a regeneração do alfa tocoferol um importante agente lipossolúvel (HEINONEN e PIIRONEN, 1991). Os tocoferóis ou vitamina E são varredores de radicais peroxil sendo, portanto os inibidores mais importantes da peroxidação lipídica em animais (FERREIRA e MATSUBARA, 1997).

Os tióis de baixo peso molecular como a glutationa e cisteína são importantes antioxidantes na manutenção da integridade celular (MEISTER et al., 1979). A membrana do eritrócito é rica em ácidos graxos poliinsaturados, sendo alvo primário para reações envolvendo radicais livres CHIHUAILAF et al., 2002; HALLIWELL e GUTTERIDGE, 2007). O que os torna ainda vulneráveis aos danos oxidativos é presença de ferro intracelular que pode catalisar estas reações (CLEMENS e WALLER, 1987). A diminuição na concentração de NPSH é um forte indicativo de estresse oxidativo nos eritrócitos (MARI et al., 2009).

Neste contexto, tentamos conhecer a influência da infecção por *B. bigemina* na atividade das enzimas dos sistemas colinérgico e da infecção por *B. bovis*, *B. bigemina* e *A. marginale* nos parâmetros de estresse oxidativo de bovinos, com intuito de contribuir no esclarecimento da patogênese da Tristeza Parasitária Bovina, assim como servir de base para posteriores estudos referentes ao uso de antioxidantes na alimentação animal para evitar a gravidade das lesões causadas por estes parasitas.

ARTIGO I: Publicado no periódico *Comparative Clinical Pathology*

BRIEF COMMUNICATION

Lipid peroxidation and decrease on the activities of antioxidant enzymes in experimental infection by *Babesia bovis* in cattle

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Abstract

Babesia bovis is one of the causative agents of bovine babesiosis, a disease with high morbidity and mortality in susceptible populations. The aim of this study was to evaluate the occurrence of oxidative stress in cattle experimentally infected with attenuated *B. bovis*. For that eight healthy cattle were used divided into two groups: animals infected with *B. bovis* (n=4; group A) and non-infected animals (n=4; group B). Blood samples of all animals were collected at 0, 7, 11 and 15 days post-infection (DPI) for red blood cells (RBCs) count, and measurement of TBARS levels in serum (lipid peroxidation) and activity of antioxidant enzymes in whole blood (catalase - CAT, and superoxide dismutase - SOD). The parasitemia was determined by blood smear evaluation and conventional PCR for *B. bovis*. Blood smears were negative throughout the experiment, however infection was confirmed by PCR positive for *B. bovis* at 15 DPI. A slight reduction on RBCs count was observed in cattle of group A at 11 and 15 DPI ($P<0.05$). The same animals showed an increased level of TBARS ($P<0.05$) at 11 DPI, suggesting lipid peroxidation; whilst the activities of CAT and SOD decreased ($P <0.05$) at 7 and 15 DPI, respectively. Our data support the occurrence of an oxidative/antioxidant imbalance in cattle infected with *B. bovis*.

Keywords: Babesiosis, TBARS, CAT, SOD.

Introduction

Bovine babesiosis is caused by the piroplasms *Babesia bovis* and *Babesia bigemina*, both transmitted solely by the tick *Rhipicephalus microplus* in Brazil. The disease has high morbidity and mortality in susceptible populations if not treated (Riet-Correa et al., 2001; Berenguer, 2006). In addition, clinical signs may include fever, anemia, jaundice, anorexia, prostration, hemoglobinuria, abortions, weight loss and reduction in milk production, causing great damage to livestock (Martins and Corrêa 1995; Bowman 2010).

Oxidative stress in the pathogenesis of *B. bigemina* is directly related to the parasite load, lipid peroxidation of the erythrocyte membrane, osmotic fragility, and intravascular hemolysis (Morita, 1996; Saleh, 2009; Harvey, 2001). The process of lipid peroxidation is measured by malondialdehyde levels (MDA) analyzed by quantifying the levels of thiobarbituric acid reactive substances (TBARS) (Esterbauer 1993; Halliwell and Gutteridge 2007). Pathological changes can lead to the formation of hydrogen peroxide and reactive oxygen species (Cohen and Hochstein 1964). To counteract these oxidative lesions, the body has endogenous antioxidant defenses, such as superoxide dismutase (SOD) and catalase (CAT) enzymes. SOD and CAT act by reducing the levels of reactive oxygen species (ROS), characterized by superoxide anion and hydrogen peroxide, and thereby inhibit the formation of hydroxyl radical, which is toxic to cells and tissue (Halliwell and Gutteridge 2007). In bovine babesiosis caused by *B. bovis* these antioxidant enzymes have not been evaluated. Therefore, the aim of this study was to evaluate the occurrence of oxidative stress in cattle experimentally infected with *B. bovis* by measuring lipid peroxidation and the activity of antioxidant enzymes (CAT and SOD).

Material and Methods

Animals

This study used eight cattle (female, 6 to 8 months old), Aberdeen Angus breed, selected from a farm free of ticks and with animals tested seronegative for *Anaplasma* spp, *B. bigemina*, *B. bovis*, infectious bovine rhinotracheitis (IBR), bovine viral diarrhea (BVD) and *Leptospira* spp. The animals were fed alfalfa hay (*Medicago sativa*) and water ad libitum. They were kept in individual pens with insect protection throughout the experimental phase. The animals were divided into two groups, four cattle infected with *B. bovis* (Group A) and four non-infected (group B). The project was approved by the

Committee of Ethics and Animal Welfare of *Instituto de Pesquisas Veterinárias Desidério Finamor* (IPVDF; Protocol number: 01/2011).

Inoculation

On day 0 of the experiment, four cattle of group A were inoculated intravenously (jugular vein) with approximately $1x10^8$ erythrocytes parasitized with *B. bovis* (attenuated strain Bbov IPV-1986; used to vaccines in IPVDF). The four animals on group B (control) received 5.0 mL of sterile saline by the same route.

Collection of blood samples

The animals were restrained in appropriate trunks for cattle, and then blood samples were collected on days 0, 7, 11 and 15 post-infection (DPI) through the jugular vein with the aid of a vacutainer system. A blood aliquot (2.0 mL) was placed into tubes with anticoagulant (EDTA) for holding the erythrocyte count, the evaluation of parasitemia, and SOD and CAT activity in whole blood. Another aliquot of 2.0 ml was placed into red-top tubes (without anticoagulant) to obtain serum for TBARS analysis.

Parasitemia estimation and counting of total erythrocytes

To monitor parasitemia, blood smears with blood collected from the jugular vein were prepared, stained with *Panóptico Rapido* kit, and evaluated for the presence of the parasite under a light microscope at 100x magnification. Count of red blood cells (RBCs) was performed on automated hematology counter (BC 2800vet®).

Lipid peroxidation

Lipid peroxidation was estimated in plasma by measurement of thiobarbituric acid reactive substances (TBARS) according to the method previously described (Jentzsch et al., 1996), using 1 % phosphoric acid and 0.6 % thiobarbituric acid (TBA). The reaction product was measured spectrophotometrically at 532 nm and the results were expressed as nmol of MDA/mL of serum.

CAT and SOD activities

Quantification of CAT activity in whole blood was carried out according to the method described by Nelson and Kiesow (1972) with modifications. An aliquot (0.02 mL) of blood (diluted 1:10 with saline) was homogenized in 0.910 mL of 50 mM potassium phosphate buffer pH 7.0. The spectrophotometric determination was initiated by the addition of 0.07 mL of 0.3 M H_2O_2. The change in absorbance at 240 nm was measured for 2 min. CAT activity was calculated using the molar extinction coefficient and the results were expressed as nmol of CAT per milligram of protein.

SOD activity in whole blood was measured based on the inhibition of O_2^- reaction with adrenalin as described by McCord and Fridovich (1969). A unit of SOD is defined as the amount of enzyme that inhibits by 50% the speed of epinefrin oxidation. It leads to the formation of the red-colored product, adrenochrome, which is detected by a spectrophotometer. SOD activity is determined by measuring the speed of adrenochrome formation, observed at 480 nm, in a reaction medium containing 50 mM glicine–NaOH pH 10 and 1.0 mM adrenalin. The results were expressed as UI SOD per milligram of protein.

Molecular analysis (conventional PCR)

The DNA was extracted from 200 µL of whole blood (sodium citrate tubes) collected at 15 DPI using a commercial kit (Invitrogen Pure Link Genomic DNA) according to the manufacturer's instructions. The detection of *B. bovis* by cPCR was performed according to Ybañez et al. (2013), using specific primers for the RAP-1 gene (BbovF 5'-CACGAGGAAGGAACTACCGATGTTGA-3' and BbovR 5'-CCAAGGAGCTTCAACGTACGAGGTCA-3'). The expected size of the amplified fragment is 252 bp. A strain of *B. bovis* (Bbov IPV-1986) was used as reaction control of the cPCR assay. Milli-Q sterile water was used as negative control of the assay.

Statistical analysis

Data of RBCs, TBARS, CAT and SOD were first evaluated by descriptive analysis; measures of central tendency and dispersion were computed. Further, all variables were submitted to Shapiro and Wilk's test. Since most of the data did not meet the assumption of parametric testing, the nonparametric test for two independence groups Mann–Whitney test was used. Results were considered statistically different when P-value was <0.05. The Spearman correlation was also conducted to identify the relation between RBCs variable and TBARS levels.

Results

Cattle experimentally infected with attenuated *B. bovis* showed extremely low parasitemia, not observed in blood smears, but detected by cPCR specific for the parasite. The animals showed no apparent clinical signs of the disease. Results of RBCs count, TBARS levels, and SOD and CAT activities are presented in Table 1. At 11 and 15 DPI, a slight but significant reduction in the erythrocytes number in the infected animals compared

to the non-infected ones was observed ($P<0.05$). TBARS levels increased in the serum of infected animals on 11 DPI ($P<0.05$), and showed a tendency to increase on 15 DPI ($P=0.062$). A significant decrease in CAT activity was observed on 7 DPI ($P<0.05$), as well as a tendency to decrease in SOD activity ($P=0.071$). At 15 DPI, SOD activity decreased significantly in cattle infected with *B. bovis* ($P<0.05$), while CAT activity showed a trend to decrease ($P=0.075$). A negative correlation ($P<0.01$) was observed between RBCs and TBARS levels on 11 DPI ($r=-0.69$) and 15 DPI ($r=-0.56$).

Discussion

The animals experimentally infected with *B. bovis* showed low parasitemia, which may be the cause of small changes observed in RBC and oxidative/antioxidant status. The attenuated strain used in this study causes very low parasitemia, which may explain the asymptomatic infection. In addition, the slight decrease on RBCs count observed in the infected group does not characterize anemia, the main clinical sign of babesiosis (Yokoyama et al. 2006; Saleh 2009).

Oxidative damage in red blood cells causes changes in their structure and function, causing precipitation and denaturation of the hemoglobin, methemoglobin formation is markedly increased in early anemia (Esmaeilnejad et al. 2012). The negative correlation between RBC count and levels of TBARS, as observed in this study, may be one of the factors involved in the reduction of red blood cell values with consequent reduction of the lifetime of erythrocytes.

Despite the asymptomatic infection, cattle in the group A had a mild lipid peroxidation (increased level of TBARS), as previously shown in *B. bovis* infection (Commins et al. 1988). Oxidative stress has been described in animals with babesiosis as a form of protection against the parasite since the lipid peroxidation described in infections

may harm the membranes, nucleic acid and proteins of these parasites causing their death (Commins et al. 1988; Stich et al. 1998; Kumar et al. 2006; Saleh 2009). However, when the oxidative stress is excessive it contributes to the pathogenesis of the disease injuring host cells and aggravating the clinical and pathological changes (Visser et al., 1995).

In this study, the antioxidant enzymes SOD and CAT had their activities decreased in some time points of the experiment. In another study with *B. bovis*, evaluating other antioxidant variables similar results were observed, i.e. a decrease in the antioxidant vitamin E and in sialic acid activities (Commins et al. 1988). The CAT and SOD are important enzymes to maintain oxidative balance and protect cells such as erythrocytes. However, both enzymes have been reported with reduced activity in cattle infected with *Theileria annulata* (Asri-Reazei and Dalir-Nagadeh 2006) and sheep infected with *B. ovis* (Esmaeilnejad et al. 2012), similar to the findings of our study. This reduction in the activity of antioxidant enzymes in ruminants with babesiosis is challenging to explain since an increase would be more likely to occurr in the presence of lipid peroxidation and/or protein oxidation.

In summary, cattle experimentally infected with *B. bovis* developed subclinical infection and mild reduction in RBCs count. In addition, the infection caused mild lipid peroxidation with a decrease in the activity of antioxidant enzymes. Therefore, based on our data the experimental infection with the attenuated strain of *B. bovis* did not cause oxidative stress, but an oxidative/antioxidant imbalance in the infected cattle.

Acknowledgement

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Table 1. Median and standard deviation of red blood cells (RBCs), levels of thiobarbituric acid reactive substances (TBARS) in serum, and catalase (CAT) and superoxide dismutase (SOD) activity in total blood of cattle experimentally infected with *Babesia bovis*.

Variables	Groups	Day 0	Day 7	Day 11	Day 15
RBCs ($x10^6/\mu L$)	A: Infected	11.35±2.32	9.67±2.03	7.25±1.85	9.25±1.56
	B: Uninfected	11.28±1.25	11.20±1.32	11.37±1.54	11.39±1.03
	*P**	>0.05	>0.05	<0.05	<0.05
TBARS (nmol of malondialdehyde/mL)	A: Infected	12.0±3.48	12.8±3.33	14.3±1.12	13.7±1.25
	B: Uninfected	11.3±2.41	10.6±3.74	12.1±1.79	11.0±1.87
	*P**	>0.05	>0.05	<0.05	=0.062
CAT (nmol of CAT/ mg of protein)	A: Infected	6.99±1.57	5.15±0.89	4.28±2.24	4.12±1.25
	B: Uninfected	5.40±1.87	7.85±1.56	5.12±1.55	5.32±1.87
	*P**	>0.05	<0.05	>0.05	=0.075
SOD (UI SOD/mg of protein)	A: Infected	13.39±1.08	10.70±0.45	10.65±0.87	8.86±0.57
	B: Uninfected	13.04±0.94	11.55±1.06	9.63±0.75	11.78±1.14
	*P**	>0.05	=0.071	>0.05	<0.05

* Median with P<0.05 statistically different between groups.

ARTIGO II: submetido a publicação no periódico *Research in Veterinary Science*:

Experimental infection by *Babesia bigemina* in cattle: influence of disease on cholinesterase and oxidative balance

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ABSTRACT

The objective of this study was to assess the influence of an asymptomatic experimental infection by *Babesia bigemina* on cholinesterases and biomarkers of oxidative imbalance. For this purpose, eight naïve animals were used: four uninfected controls and four infected with an attenuated strain of *B. bigemina.* Blood samples were collected on days 0, 7 and 11 post-inoculation (PI). Parasitemia was determined by blood smear evaluation, showing that the infection by *B. bigemina* resulted in mean 0.725 and 0.025% on day 7 and 11 PI, respectively. The activities of acetylcholinesterase, butyrylcholinesterase and catalase were lower, while levels of thiobarbituric acid reactive substances and superoxide dismutase activity were higher in infected animals, when compared with the control group. This attenuated strain of *B. bigemina* induced an oxidative stress condition, concomitant with the parasitemic peak, and altered the cholinesterase´s activity in infected and asymptomatic cattle.

Keywords: Babesiosis; acetylcholinesterase; catalase; superoxide dismutase; lipid peroxidation.

1. Introduction

Bovine babesiosis is caused by the protozoa *Babesia bovis* and *B. bigemina*, both transmitted solely by the tick *Rhipicephalus* (*Boophilus*) *microplus*, in Brazil (Berenguer, 2006; Riet-Correa et al., 2001). Babesiosis usually causes high morbidity and mortality in susceptible populations, with animals clinically coursing with fever, anemia, jaundice, anorexia, prostration, weight loss, reduction in milk production, hemoglobinuria, abortions and death. Therefore, it causes major damage to livestock (Bowman, 2010; Fonseca and Braga, 1924; Kessler et al., 1998; Martins and Corrêa, 1995; Soulsby and Mönnig, 1968).

The disease´s severity is related mainly to the hemolytic anemia caused by the rupture of erythrocytes during multiplication of the parasite, increased erytrophagocytosis by activated macrophages, production of autoantibodies, increase in membrane permeability (Alkhalil et al., 2007; Goes et al., 2007; Ristic et al., 1981), and methemoglobin formation, osmotic fragility and cell destruction, usually caused by the erythrocyte´s oxidation (Harvey, 2001). After an acute infection by *B. bigemina*, animals develop low parasitemia and remain asymptomatic carriers for up to 2 years (Callow and Hoyte, 1961; Mahoney et al., 1973).

In subclinical cases, cell lesions can be detected by low-grade inflammatory markers such as cholinesterase (Das, 2012) and biomarkers of oxidative stress (Halliwell and Gutteridge, 2007). The cholinergic system is a major modulatory pathway in the central nervous system (CNS), composing an essential part of several vital functions, such as regulation of cerebral blood flow (Mesulam et al., 2002; Rang and Dale, 2007). Acetylcholine (ACh) is the main component of the cholonergic system and is a molecule mainly hydrolyzed by acetylcholinesterase (AChE), a widely distributed enzyme in the CNS, but is also found in erythrocytes, platelets and lymphocytes (Çokuğraş, 2003; Silva, 1998). In blood, AChE activity is considered a good marker of CNS peripheral changes, especially since it presents functional properties similar to those observed into the synaptic cleft

(Thiermann et al., 2005). Additionally, butyrylcholinesterase (BChE) may also hydrolize ACh, especially when AChE is inhibited (Li et al., 2006). The increase in the activity of AChE and BChE leads to a decreasein the levels of ACh, thus, reducing its anti-inflammatory effects (Rao, 2004). Therefore, AChE and BChE activities are considered intrinsic regulators of inflammation (Anglister et al., 2008; Das, 2012).

Furthermore, there may be an involvement of oxidative stress mechanisms acting in the pathogenesis of babesiosis, since the severity of infection by *B. bigemina* is directly related to the parasite load and lipid peroxidation of the erythrocyte membrane, along with the formation of methemoglobin, factors that normally aggravate the osmotic fragility, as well as the intravascular hemolysis, situations that increase the elimination of these cells by the spleen (Harvey, 2001; Morita et al., 1996; Saleh, 2009). Lipid peroxidation generates hydrocarbons and aldehydes, such as malondialdehyde (MDA). It plays an important role on cell damage, especially at high concentrations. Thus, its assessment is important for the determination of Thiobarbituric Acid Reactive Substances (TBARS) (Esterbauer, 1993; Halliwell and Gutteridge, 2007).

The activated cells of the phagocytic system produce reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are responsible for the degeneration of babesias within the erythrocyte (Johnson et al., 1996). A mechanism causing the production of ROS is catabolism of polyamines by polyamine oxidase, which is present in high levels in bovine serum (Johnson et al., 1996). However, overproduction of ROS may cause serious damage to mammalian host cells creating mutations, inactivation of cellular DNA, and may initiate lipid peroxidation of cell membranes (Ferreira and Matsubara, 1997; Halliwell and Gutteridge, 2007). In order to counteract these oxidative damages, the organism has antioxidant defenses, consisting of superoxide dismutase (SOD) and Catalase (CAT)

enzymes. SOD and CAT induce a reduction on the levels of ROS (Halliwell and Gutteridge, 2007).

Since cholinesterases regulate the inflammatory immune response, this comprises a fundamental defense barrier against hemoparasites. Therefore, the aim of this study was to assess the involvement of AChE and BChE, as well as the biomarkers of oxidative stress in cattle experimentally infected with *B. bigemina*, at different time points during an asymptomatic infection.

2. Material and Methods

2.1- Animal model:

Eight female Aberdeen Angus crossbred bovines, ages 6 to 8 months were selected from a rural property known to be naturally free of ticks. They were seronegative for *Anaplasma marginale, B. bigemina* and *B. bovis*, Infectious Bovine Rhinotracheitis (IBR), Bovine Viral Diarrhea (BVD) and *Leptospira* spp . They were fed alfalfa hay, received water *ad libitum*, and were housed in individual pens which provided protection from insects and ticks throughout the experimental period. The animals were divided into two groups: four animals infected with an attenuated strain of *B. bigemina* (Bbig791), and four uninfected controls.

The experimental protocol was submitted and approved by the Board of Ethics and Animal Welfare of *Instituto de Pesquisas Veterinárias Desidério Finamor*, protocol number 01/2011 – *IPVDF*.

2.2- Parasite Inoculation

On day zero, all the animals from the infected group were inoculated intravenously (jugular vein), with approximately $1x10^6$ erythrocytes parasitized by the attenuated *B.*

bigemina strain. The control group was treated with 5 mL of sterile saline solution by the same route. *B. bigemina* strain (Bbig791) was attenuated at *Instituto de Pesquisas Veterinárias Desidério Finamor* through 7 passages in *naïve* splenectomized calves and was stored in liquid nitrogen experimental uses.

After inoculation, in order to establish the onset of parasitemia, blood smears were prepared daily using the Romanowski staining method from whole blood.

2.3- Sample collection

Blood samples were drawn on 0, 7 and 11 days post-infection (PI), by jugular puncture using a vacutainer tube. Blood was stored in 4.5 mL tubes containing EDTA (for complete blood count, AChE activity and PCR), sodium citrate (for assessment of CAT and SOD) and without anticoagulant (for evaluation of TBARS and BChE). Blood counts were performed within 4 hours after collection and the samples were frozen in 1.5 mL aliquots at -18 °C for up to 60 days.

2.4- Hematological evaluations

The whole blood samples were used to evaluated the red blood counts (RBC), hematocrit (Ht), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), plasma protein concentration, and total leukocytes (WBC) followed by leukocyte differential. Blood smears were prepared and stained with *Romanowski* method for differential leukocyte count, cell morphological and parasitemia evaluations, estimated by counting 1000 erythrocytes. The determination of plasma fibrinogen was performed according to Schalm et al. (1975). RBC and Hb were performed using an automated hematology counter (BC 2800vet), leukocyte counts were performed manually, according to the technique described by Schalm et al. (1975). MCV

and MCHC values were determined by indirect calculations, and differential leukocyte count was performed according to the technique described by Thrall (2012).

2.5- AChE activity

In order to assess AChE activity, the EDTA-whole blood, was centrifuged for 10 min at 1,000g. The plasma was discarded. The erythrocyte sediment was washed three times with tenfold isotonic NaCl solution. After each procedure, the erythrocyte–saline mixture was centrifuged at 1,000g for 10 min. The erythrocytes obtained were used to assess AChE activity. AChE activity was determined using the method of Ellman et al. (1961) modified by Worek et al. (1999). To achieve temperature equilibration and complete reaction of sample matrix sulfhydryl groups with 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB), the mixture was incubated for 10 min prior to the addition of substrate. Enzyme activity was corrected for spontaneous hydrolysis of the substrate and DTNB degradation. The activity of butyrylcholinesterase (BChE; EC 3.1.1.8) was inhibited by ethopropazine. The AChE activity was measured at 436 nm and calculated from the quotient between the AChE activity and the hemoglobin content (Hb). Hb was determined using the Zijlstra-modified solution. Results were expressed as mU/umolHb.

2.6- BChE Activity

The BChE enzymatic assay was determined in serum by a modification of the spectrophotometric method of Ellman et al. (1961). The reaction mixture (2 ml final volume) contained 100 mM potassium phosphate buffer, pH 7.5, and 1.0 mM DTNB. The method is based on the formation of the yellow anion. The absorbance was measured at 412 nm during the 2 minutes of incubation at 25 °C. Enzyme activity was expressed in μmol BuSCh/h/mg of protein.

2.7- Lipid peroxidation

Lipid peroxidation was estimated in serum by the measurement of TBARS according to the method of Jentzsch et al. (1996). The reaction product was measured spectrophotometrically at 532 nm and the results were expressed as nmol MDA/mL of serum.

2.8- CAT and SOD activities

Superoxide dismutase activity in whole blood was performed according to the method of Misra and Fridovich, 1972. In this method, SOD present in the sample competes with the detection system for radical superoxide. A unit of SOD is defined as the amount of enzyme that inhibits the speed of oxidation of adrenalin by 50%. The oxidation of adrenalin leads to the formation of the colored product, adrenochrome, which is detected by spectrophotometer. SOD activity is determined by measuring the speed of adrenochrome formation, observed at 480 nm, in a reaction medium containing glicine–NaOH (50 mM, pH 10) and adrenalin (1 mM). The SOD activity was expressed in U SOD/mg protein.

The determination of the CAT activity in whole blood was carried out in accordance with a modified method of Nelson and Kiesow (1972). This assay involved the change in absorbance at 240 nm due to CAT-dependent decomposition of hydrogen peroxide. An aliquot (0.02 mL) of blood was homogenized in potassium phosphate buffer, pH 7.0. The spectrophotometric determination was initiated by the addition of 0.07 mL in an aqueous solution of hydrogen peroxide 0.3 mol/L. The change in absorbance at 240 nm was measured for 2 min. The CAT activity was calculated using the molar extinction coefficient (0.0436 cm2/lmol) and the results were expressed as nmol/mg protein.

2.9- PCR

Genomic DNA was extracted from 200µl of EDTA-whole blood using a DNA extraction kit (InvitrogenPure link Genomic DNA) while following the manufacturer's instructions. The molecular detection of *B. bigemina* was performed according to Figueroa et al. (1992), using the primers BbigF (5'-CATCTAATTTCTCTCCATACCCCTCC-3') and BbigR (5'-CCTCGGCTTCAACTCTGATGCCAAAG-3'). The total reaction was 25 µl and was comprised of 2.5 µL PCR buffer (Invitrogen); 1.5 mM of $MgCl_2$; 0.2 mM of each dNTP; 0.2 µM of each primer, 0.5 units of Taq DNA polymerase (Platinum Taq DNA Polymerase, Invitrogen) and 50 ng of genomic DNA. Amplifications were performed in a thermocycler (Veriti 96 WellThermalCycler - AppliedBiosystems) The protocol consisted of an initial denaturation at 94 °C, for 2 minutes, followed by 35 cycles of 94 °C for 30 seconds, 61 °C for 30 seconds, and 72 °C for 30 seconds, ending with a final extension at 72 °C for 7 minutes. The products of amplification were separated by electrophoresis using a 1% ultra pure agarose gel (Invitrogen), and stained with ethidium bromide (0.025 µl/mL). Amplification products were visualized and photographed under ultraviolet transilluminator. In order to estimate the size of the amplified fragments, a 50 bp DNA Ladder (Invitrogen) was used. *B. bigemina* strain BBig IPV-1985, was used as a positive control to verify the presence of a 277bp fragment . Sterile water was used a reaction control. Control animals were checked by PCR to ensure they were negative for *B. bigemina* at the beginning and upon completion of the experiment.

2.10- Data analysis

The results were evaluated using an Analysis of Variance (ANOVA) of repeated data, which compared the infected group to the control group at different time points. Data analysis was performed using GraphPadPrism 5.

3. Results

3.1- Parasitemia evaluation

It was identified forms compatible with *B. bigemina* from day 6 PI, verifying an average parasitemia of 0.725% on day 7 PI. However, 11 days PI, one animal remained positive on blood smear evaluation (Table 1). Molecular analysis confirmed all the infected animals were positive for *B. bigemina*, on both days tested. The animals were asymptomatic throughout the experiment.

3.2- Hematological analysis

The hematocrit and hemoglobin levels decreased in infected animals when compared with controls on day 11 PI ($P<0.01$). However, levels of fibrinogen increased on days 7 and 11 PI in infected animals compared with the control group (Table 1), WBC assessment did not show a significant difference between infected and control groups, just a tendency of leukocytes reduction was observed due of a decrease in lymphocytes count (Table 1). On day 7 PI, a reduction of platelets was observed in infected animals.

3.3- AChE and BChE activities

A lowered activity of AChE and BChE on day 7 PI for the infected animals when compared with the control group was observed (Figure 1A and B). However, on day 11 PI there was no difference between the groups.

3.4- TBARS levels

On day 7, TBARS levels were significantly higher in infected animals compared with uninfected animals ($P<0.01$). No significant difference of TBARS levels was found on day 11 PI ($P>0.05$) (Figure 2a). Assessment of antioxidant enzymes showed reduced CAT

activity on day 7 PI for the infected group compared with the control, while SOD activity showed an increased activity. (Figure 2B and C).

4. Discussion

Animals experimentally infected with an attenuated *B. bigemina* strain were asymptomatic throughout the experiment, but changes in their cholinesterases activity were observed. An oxidative stress imbalance concomitant with a parasitemia peak was also observed.

The hemogram results showed Ht and Hb levels were decreased, followed by an increase in fibrinogen concentration. It is well known that hemolytic anemia is a characteristic of bovine babesiosis, since these protozoa normally lead to erythrocytes disruption during their proliferation (Alkhalil et al., 2007; Ristic, 1981). During erythrocyte´s infection, it is possible that oxidative damage, autoimmune phenomena and erytrophagocytosis contribute to the development of a hemolytic anemia (Esmaeilnejad et al., 2012). Moreover, fibrinogen, an acute phase protein in infections, is highly present in inflammatory processes. In cattle and sheep, this parameter is used as an indicator of bacterial infection, inflammation or surgical trauma (Murata et al., 2004). However, no significant differences were observed in WBC parameters, despite a tendency of leukocytes reduction in infected animals. This may be due to the low parasitemia observed since the onset of clinical manifestations begins when parasitemia by *B. bigemina* is higher than 1% (Monteiro, 2010) (Table 1).

Blood cholinesterase´s activity has been investigated since it indicates the integrity and functional state of erythrocytic membrane and may serve as markers of systemic low-grade inflammation (Das, 2012). Our data represents the first study where the cholinesterase activities were assessed in bovine babesiosis. However, decreases in AChE and BChE

activities have been previously reported in other hemoparasites, such as experimental *Trypanosoma evansi* infection in cats (Da Silva et al., 2010) and in rats (Wolkmer et al., 2010). In dogs experimentally infected with *Rangelia vitalii* (molecularly related as a *Babesia* specie), an increased AChE activity in whole blood was observed, while BChE activity was reduced in serum (Da Silva et al., 2013). Reduced BChE activity was also found in the current study.

Once the infectious process is set, a reduction in cholinesterase activity could occur as a compensatory mechanism leading to an increase of ACh concentration in serum. The cholinergic anti-inflammatory pathway mediated by ACh acts by inhibiting the TNF-α and MIF (inhibitory factor of macrophage migration) (Das, 2012). In this study, the reduction of seric AChE and BChE activities on day 7 PI may have produced a decrease in ACh hydrolysis, thus inhibiting the release of IL-1, TNF-α and other pro-inflammatory agents, leading to a cholinergic system modulation (Darreh-Shori et al., 2013; Reale et al., 2013), change proportional to the peak of parasitaemia. It is a situation also observed in the advanced stage of certain disease such as Alzheimer's, diabetes mellitus, hypertension, hyperlipidemia and insulin resistance in humans (Das, 2012). On the other hand, the low activity of AChE and BChE could also be associated with an increase in lipid peroxidation, as observed in this study. Lipid peroxidation can easily lead to cell damage, thus interfering with the activity of these enzymes anchored on the cell membrane (de Carvalho Corrêa et al., 2008; Gonçalves et al., 2010; Gutierres et al., 2012).

Cholinesterases isoforms, which are soluble and are found anchored to biological membranes, are extremely sensitive to lipid peroxidation. Damage to the membrane destabilizes its optimal conformation, reducing its catalytic activity. Lipid peroxidation is responsible for reducing the activity of membrane enzymes and, the change of the cholinergic signaling. It usually leads to cytotoxity, as well as inflammatory events that can

be deleterious to the cells (Gutierres et al., 2012; Marisco et al., 2013; Pimentel et al., 2013; Spanevello et al., 2009; Zanini et al., 2013). Our data shows that disorders of the cholinergic system seem to be associated with cytotoxic and inflammatory events. AChE is produced by lymphocytes which are cells normally involved in immune response regulation (Kawashima and Fujii, 2000). Therefore, a reduction of circulating lymphocytes could result in a reduction of AChE. In this study, there was a decrease in the lymphocytes count, along with a reduction in AChE levels.

For many years the involvement of free radicals in the pathogenesis of parasitic infections has been the subject of research, as in studies with visceral leishmaniasis in humans (Biswas et al., 1997), *Babesia bovis* infection in cattle (Stich et al., 1998), *Plasmodium falciparum* in humans (Harwaldt et al., 2002), *Hepatozoon canis* in dogs (Kiral et al., 2005), *Erlichia canis* and *Babesia gibsoni* in dogs (Kumar et al., 2006), *Babesia gibsoni* in dogs (Chaudhuri et al., 2008), *Theileria annulata* in cattle (Asri Rezaei and Dalir-Naghadeh, 2006), *Babesia bigemina* in cattle (Saleh, 2009), *Trypanosoma evansi* in camels (Saleh et al., 2009), *Babesia canis* in dogs (Crnogaj et al., 2010) and *Babesia ovis* in sheep (Esmaeilnejad et al., 2012). The main targets of reactive species are the polyunsaturated fatty acids on the phospholipid membranes which results in disruption of cell structure and function, beyond the production of MDA (Patterson and Leake, 1998). Erythrocytes are highly susceptible to oxidative damage, since they carry large amounts of polyunsaturated fatty acids on their membrane. Thus, they are continuously exposed to high concentrations of oxygen and iron, a powerful transition metal catalyst (Kumar et al., 2006; Sahoo et al., 2001; Saleh, 2009). One of the most important findings in this study was the reduced cholinesterase activity and the increased TBARS levels on day 7 PI, in infected animals. Enhanced levels of TBARS in erythrocytes of cattle have been reported in *B. bovis* infection (Commins et al., 1988), *B. bigemina* (Saleh, 2009) and bovine theileriosis (Asri Rezaei and

Dalir-Naghadeh, 2006; Shiono et al., 2001), corroborating our findings. The experimentally infected animals in the current study did not show clinical signs of disease, unlike other studies whose animals did show clinical signs.

Oxidative damage in red blood cells causes structural and functional changes, leading to hemoglobin precipitation, denaturation and methemoglobin formation. Methemoglobin formation is markedly increased in early anemia (Esmaeilnejad et al., 2012). *Babesia* is sensitive to oxidative stress because lipid peroxidation can damage membranes, nucleic acids and proteins, causing mortality of these parasites (Kiral et al., 2005; Kumar et al., 2006; Saleh, 2009; Stich et al., 1998), For this reason, oxidative products when produced in optimal amounts, contribute to the immune response against parasites (Kiral et al., 2005). However, this threshold is narrow, because when the oxidative products are in excess, they contribute to the disease´s pathogenesis by increasing cell damage (Visser et al., 1995).

Other important markers for assessing oxidative stress balance, are the assessment of SOD and CAT activities in whole blood. SOD plays an important role in protecting erythrocytes against oxidative damage, which could explain its increase on day 7 PI. It is important to emphasize that SOD neutralizes ROS, and the decrease in its activity has been reported in cattle with *T. annulata* (Asri Rezaei and Dalir-Naghadeh, 2006), in sheep with *B. ovis* (Esmaeilnejad et al., 2012), and camels with *T. evansi* (Saleh et al., 2009), However, in our study, an increase in SOD activity was found in infected cattle. Chaudhuri et al. (2008) also reported an enhancement in SOD activity in erythrocytes of dogs naturally infected with *B. gibsoni*. This was probably due to increased activity and expression of SOD by endogenous regulatory mechanisms, in order to prevent oxidative damage by superoxide radicals generated during parasite multiplication into the erythrocytes.

In this study, lowered activity of CAT in whole blood was observed. Similar findings in cattle with *T. annulata* (Asri Rezaei and Dalir-Naghadeh, 2006), and in sheep infected

with *B. ovis* (Esmaeilnejad et al., 2012) have been reported. The reduction in CAT activity may have occurred due to a large amount of substrate supplied by SOD activity (Figure 2C) causing depletion of CAT.

Based on the results, cattle infected with *B. bigemina* underwent an oxidative stress process concomitant with the reduction of cholinesterase activity. These results have influenced the pathogenesis of this infection even under lower grade parasitemia and without clinical signs. Therefore, these parameters require further investigation in asymptomatic animals in order to better understanding babesiosis, as well as assess possible markers for early diagnosis of this parasitic disease.

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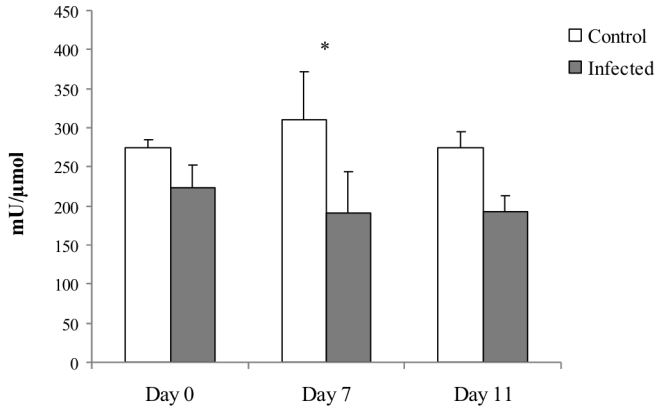
Table 1: Means and standard errors of hemogram, total plasma proteins, fibrinogen and parasitemia in cattle experimentally infected with *Babesia bigemina***.**

Variables	Day 0 PI	Day 7 PI	Day 11 PI
RBC ($x10^6$/µL)			
Controls	9.91±0.41	10.48±0.58	10.49±0.50
Infected	11.91±0.83	10.90±0.63	9.01±0.65
Hematocrit (%)			
Controls	34.5±1.47	32.25±1.02	35.5±0.67**
Infected	37.67±0.88	30.50±0.64	28.67±2.03**
Hemoglobin (g/dL)			
Controls	11.50±0.58	11.28±0.54	12.18±0.20**
Infected	12.30±0.61	9.95±0.25	9.40±0.72**
MCV (fL)			
Controls	34.78±0.68	31.03±1.96	34.03±1.48
Infected	29.64±1.16	28.19±1.31	31.87±1.00
MCHC (g/dl)			
Controls	33.36±0.34	35.17±2.44	34.32±0.46
Infected	32.61±0.89	32.62±0.49	32.77±0.39
Plasma Proteins (g/dL)			
Controls	8.45±0.15	7.95±0.25	8.35±0.26
Infected	8.87±0.37	8.85±0.40	8.93±0.54
Fibrinogen (mg/dL)			
Controls	400±0	400±81.65**	300±57.74**
Infected	667±66.67	950±95.74**	800±115.5**
WBC ($x10^3$/µL)			
Controls	14.575±2.96	11.250±0.51	13.046±2.55
Infected	15.967±4.81	6.625±0.68	8.155±1.68
Segmented Neutrophils ($x10^3$/µL)			
Controls	6.433±0.61	2.857±0.65	3.949±210.0
Infected	7.958±4.05	2.718±0.80	3.598±1.55
Lymphocytes ($x10^3$/µL)			
Controls	7.356±2.91	7.263±0.95	8.170±1.88
Infected	7.817±0.90	3.537±0.18	4.134±0.94
Monocytes ($x10^3$/µL)			
Controls	629±234.9	563±108.4	316±65.9
Infected	64±64.0	329±220.6	423±82.7
Eosinophils ($x10^3$/µL)			
Controls	158±90.96	567±100.3	579±143.6
Infected	128±128	0	0
Platelets ($x10^3$/µL)			
Controls	634.7±75.72	726.7±96.27	433.0±92.12
Infected	884.0±49.5	436.0±44.77	301.9±246.1
Parasitemia (%)			
Controls	0	0**	0
Infected	0	0.725±0.36**	0.025±0.025

Where: PI=post-infection, RBC= red blood cells, MVC= mean corpuscular volume, MCHC= mean corpuscular hemoglobin concentration, WBC= white blood cells.

Observation: Statistical values (P) for the comparisons between infected and control animals (N=4), each day: *= P<0,05; ** =P<0,001.

A



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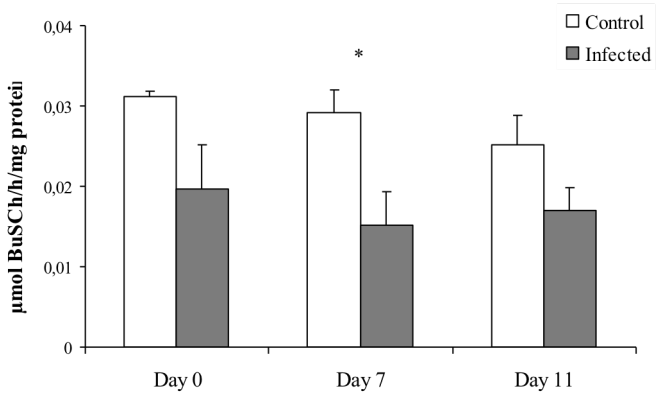
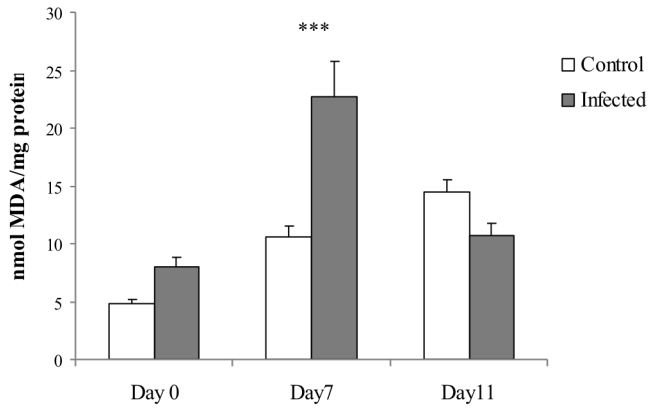


Figure 1: Activity of Acetylcholinesterase (A: AChE) in whole blood, and butyrylcholinesterase (B: BChE) in serum. Analysis performed on cattle experimentally infected with *Babesia bigemina* (N=4) on days 0, 7, and 11 post-infection (*P<0.05).

A



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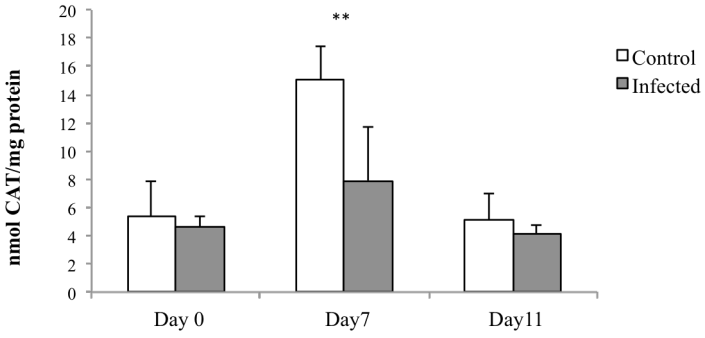

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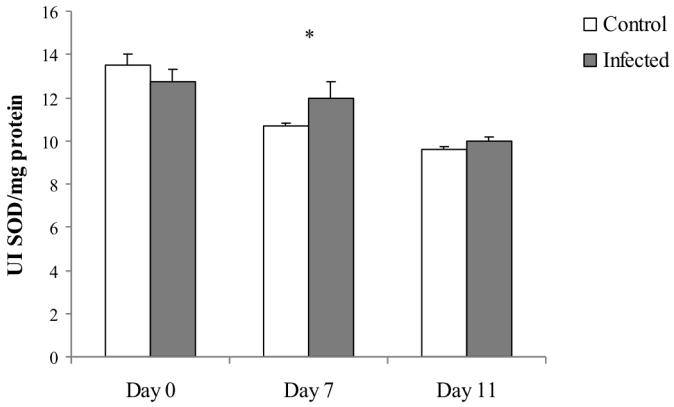

Figure 2: Levels of Thiobarbituric Acid Reactive Substances (A: TBARS) in serum, catalase activity (B: CAT) and Superoxide Dismutase (C: SOD) in whole blood. Analysis performed on cattle experimentally infected with *Babesia bigemina* (N=4) on days 0, 7, and 11 post-infection (*P<0.05; **P<0.01).

ARTIGO III: a submeter

Cattle experimentally infected by *Anaplasma marginale*: influence of splenectomy on disease, oxidative profile and antioxidant status

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Abstract

Bovine anaplasmosis is caused by the obligate intraerythrocytic bacteria *Anaplasma marginale.* These organisms are transmitted by the cattle tick, blood-sucking insects and fomites (needles, clippers, and other blood contaminated equipment). During the acute phase of infection, animals may develop fever, anemia, jaundice, and hepatosplenomegaly. The aims of this study were to quantify the bacteremia by quantitative PCR in eight naïve calves (splenectomized (n=4), and intact (n=4)) experimentally infected with *A. marginale* and to correlate these findings with markers of oxidative stress on days 0, 8, 15, 21 and 23 post infection. Lipid peroxidation was estimated by quantifying thiobarbituric acid reactive substances (TBARS), while the non-enzymatic antioxidants were assessed by erythrocyte content of non-protein thiols (NPSH). Blood counts were performed in these animals with no significant differences between groups. However, both groups after infection with *A. marginale* had a slight decrease of hematocrit, erythrocytes number and hemoglobin concentration, as well as an increase in leukocytes due to elevation of lymphocytes. Animals from both groups presented reduction on red blood cells count. The results showed progressive increase in TBARS levels and concomitant decrease in NPSH content in all animals, without significant differences between splenectomized and intact cattle. A positive correlation between bacteremia and TBARS was observed in splenectomized and intact animals. In contrast, a negative correlation was verified between bacteremia and NPSH in both groups, but the correlation was higher in splenectomized animals. A negative correlation between TBARS and NPSH levels in both groups was found, which characterizes a situation of lipid peroxidation without a non-enzymatic antioxidant response. The results of experimental infection with *A. marginale* in cattle showed that bacteremia has an impact on lipid peroxidation regardless of animals being splenectomized or not.

Key words: anaplasmosis; anemia; lipid peroxidation; antioxidants.

1. Introduction

Anaplasmosis is one of the most important tick-borne diseases in cattle. It causes significant economic losses in dairy and beef herds in tropical and subtropical areas (Gardiner et al., 1989; Guglielmone, 1995; Palmer et al., 1999; Riet-Correa et al., 2001). The etiologic agent is the obligate intraerythocytic organism *Anaplasma marginale* (de la Fuente et al., 2001). In South America, the main vector is *Rhipicephalus (Boophilus) microplus,* however, it also can be transmitted by blood sucking insects, which are less effective vectors than ticks (Bowman, 2010; Guglielmone, 1995; Valenzuela, 2004). In addition, iatrogenic or transplacental transmissions have been reported (Riet-Correa et al., 2001). In mammalian host, *A. marginale* infects red blood cells by forming a vacuole derived from the erythrocyte membrane itself, which can be visualized in the blood smear as a small intraerythrocytic basophilic inclusion, measuring 0.55-0.85 μm containing up to eight initial bodies (Corona et al., 2005).

Contrary to adult animal, which have a higher incidence and show more severe clinical signs, young animals (up to eight months old) usually develop subclinical disease due to the innate resistance (Madruga et al., 2001; Schalm et al., 1975). Acute anaplasmosis is clinically characterized by a progressive prostration, fever, weight loss, jaundice, hepatosplenomegaly, abortion and death (Jones et al., 2000). Hemolytic anemia occurs when infected erythrocytes are removed from the blood by splenic and bone marrow macrophages. Hemolysis can occur extravascularly or intravascularly resulting in anemia, which is more pronounced in splenectomized individuals (Jones et al., 1968; Ristic, 1981). During this phase of the disease, *A. marginale* can be seen in blood film and may infect as many as 50% of the red blood cells. Surviving animals become chronically infected, and develop a low cyclic bacteremia which may not been identified on blood films but is detected by molecular techniques (Hutyra et al., 1953; Schalm et al., 1975). During chronic infection, antigenic

variation occurs through modification of the outer membrane major surface proteins (MSPs). MSPs act as antigens for eliciting the immune response of the host and can be used for molecular identification (Brayton et al., 2005).

Several parasitic diseases are reported to cause subclinical inflammation with increased production of reactive species of oxygen (ROS) and/or the consumption of antioxidants, inducing oxidative stress (Chaudhuri et al., 2008; Cingi et al., 2012; Crnogaj et al., 2010; Esmaeilnejad et al., 2012; Saleh, 2009). Malondialdehyde (MDA), is an end product of lipid peroxidation, and is considered an important plasmatic biomarker for oxidative stress (Nielsen et al., 1997). To estimate MDA concentration in plasma or serum, the most popular method is based on the reaction of MDA with thiobarbituric acid (TBA), which generates a red fluorescent adduct that can be measured. This assay detects thiobarbituric acid reactive substances (TBARS) and while non-specific for MDA, it provides empirical evidence of lipid peroxidation and oxidative tissue injury (Janero, 1990; Sato et al., 1995).

Cells have defense mechanisms to inactivate ROS, among these, organic compounds containing sulfhydryl groups (SH) called thiols. Non-protein thiols are important antioxidants for the erythrocyte, helping to maintain its integrity. Because of the propensity of erythrocytes to generate radical species, it is considered a primary target for oxidative damage (Chihuailaf et al., 2002; Halliwell and Gutteridge, 1999).

Studies on oxidative stress in cattle experimentally infected with *A. marginale* are rare, moreover the effect of splenectomy in infected cattle on antioxidant status and oxidative profile is unknown. Therefore, the goal of the study described herein was to quantify the erythrocyte content of non-protein thiols and lipid peroxidation levels in the serum of calves experimentally infected with *A. marginale* (splenectomized or intact) and investigate how these markers of oxidative stress correlate with the bacteremia.

2. Material and Methods

2.1. Animals

Eight female calves, 6-8 months old, Angus breed were selected from a farm located in a naturally tick-free zone. All animals tested seronegative for *A. marginale*, *Babesia bigemina* and *B. bovis*, infectious bovine rhinotracheitis (IBR), bovine viral diarrhea (BVD), and *Leptospira* spp. three weeks before arrival at the Veterinary Research Institute Desidério Finamor, where the experiment was performed. The animals were kept in individual boxes protected from insects, fed alfalfa (*Medicago sativa*), hay and water *ad libitum*. Four animals were splenectomized 7 days before inoculation with *A. marginale* and four remained intact. The study protocol was approved by the Board of Ethics and Animal Welfare of Veterinary Research Institute Desidério Finamor under Protocol 01/2011 - CEUA / IPVDF.

2.2. Inoculation with *Anaplasma marginale*

All animals were inoculated intravenously (jugular vein) with approximately $1x10^6$ erythrocytes parasitized with *A. marginale* (day zero). The strain of *A. marginale* used as inoculum was received from UNESP-Jaboticabal/SP- Brazil (São Paulo State University) and maintained in liquid nitrogen for four months until inoculation. Blood smears of all animals were performed on days 0, 5, 10, 15, 20 and 25 post infection (PI), which were stained with Romanowsky and observed by light microscope (100x). After inoculation, the animals were observed daily for clinical alterations (e.g. elevated body temperature, prostration, and food intake).

2.3. Blood samples

Blood samples were collected on days 0, 8, 15, 21 and 23 PI from all animals by puncture of the jugular vein in vacuum system tubes. Hematologic evaluation was performed

on EDTA-whole blood, and 500 μL were used for DNA extractions using chloroform. Aliquots of 10 mL of blood were collected into red top tubes (without anticoagulant) to obtain serum for the evaluation of lipid peroxidation. Aliquots of 4.5 mL of blood were placed into tubes containing sodium citrate to measure the non-protein thiols (NPSH) content.

2.4. Hemogram

Automated hematologic evaluation was performed on the BC-2800Vet (Mindray®, China) and included red blood cell (RBC) count, hematocrit (Ht), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), leukocytes count, and platelets. The determination of the packed cell volume (PCV) followed recommendation previously described (Thrall, 2012). The total nucleated cell count was determined manually according to the technique described by Schalm et al. (1975). Blood smears were prepared and stained with aqueous Romanowski method for differential leukocyte count, morphologic evaluation (Thrall, 2012). The concentration of total plasma protein was determined by refractometry, and fibrinogen levels were determined by heating technique at 56 °C (Jain, 1993).

2.5. TBARS

Lipid peroxidation was determined as levels of thiobarbituric acid reactive substances (TBARS) according to the method described by Jentzsch et al. (1996) using 200 μL of serum mixed with 25 μL of BHT in ethanol, 200 μL of orthophosphoric acid and 25 μL of TBA reagent, incubated (for 45 minutes at 90°C), and then TBARS were extracted once with 500 μL n-butanol. Results were obtained by spectrophotometry at 535 nm and expressed in nmol of malondialdehyde/mL serum.

2.6. NPSH

Non-protein thiols (NPSH) content in erythrocytes was measured according to the Ellman method (1959) using 300 μL of whole blood collected with sodium citrate, hemolysate with triton 10% and TCA 20%, centrifuged, and the supernatant incubated (for 1 hour at 25°C in dark) and measured by absorbance (412 nm). The results were expressed as μmol/mL erythrocytes.

2.7. Bacteremia

Anaplasma marginale copy number in blood samples (days 0, 8, 15, 21 and 23 PI) was assessed using a TaqMan quantitative PCR (qPCR) based on the amplification of a fragment of the gene *msp1b* (95 bp) of this bacterium (Carelli et al., 2007). Prior to qPCR, conventional PCR (cPCR) using the primers AM456 and AM1164 for amplification of a ~730 bp fragment of the msp1b gene was performed (Carelli et al., 2007; Molad et al., 2004). The purified fragment was cloned into pGEM T cell-Vector System II (Promega, Madison - USA) and propagated in *E. coli* competent cells (JM109 Promega). Vectors containing the *msp1b* insert, tested positive by cPCR, were purified and sequenced to confirm the identity of the insert. A vector containing a confirmed fragment of the *msp1b* gene of *A. marginale* was ten-fold (10^9 to 1 DNA copy/mL) diluted and used as standard curve in the qPCR assay.

Absolute quantification assay was performed using a 7300 Real-Time PCR System (Applied Biosystems, Life Technologies Corporation). Results were analyzed using 7300 Real Time PCR System SDS software v1.2 (Applied Biosystems). The cycle threshold (Ct) of vector dilutions were plotted against the logarithm of vector copy number and used to determine the standard curve. All samples were analyzed in duplicate. The results of copy number per sample calculated based on the standard curve were divided by 3 since the qPCR

primers and probe designed for this assay are able to amplify 3 copies of the *msp1b* gene present in the genome of *A. marginale*.

2.8. Statistical analysis

Data of hemogram, TBARS, NPSH, and bacteremia were first analyzed descriptively; measures of central tendency and dispersion were computed. Further, all variables were submitted to Shapiro and Wilk's test. Since most of the data did not meet the assumption of parametric testing, the nonparametric Mann-Whitney *U* test for two independent groups, and in a same group at different time points of infection, was used. Results are presented as mean and standard error, and considered statistically different when *p*-value was <0.05. Sperman's test was applied for correlation analysis using the GraphPad Prism 5 program. The following correlations were performed: 1) bacteremia versus TBARS; 2) bacteremia versus NPSH; 3) TBARS versus NPSH.

3. Results

3.1. Clinical signs

During the daily clinical evaluation, the animals showed some characteristic signs of anaplasmosis, such as prostration and slight reduction in food consumption, however, there was no significant change between groups in the body temperature, which were within the reference interval for cattle (Thrall, 2012).

3.2. Infection control

The quantitative assessment by PCR, both groups showed high bacteremia from day 15. However, in the splenectomized group, were higher than in intact, as can be seen in Figure 1.

3.3. Hematological analysis

Erythrogram results were presented in Table 1. Statistical difference between the two groups was not observed for all erythrogram variables in the surveyed periods. However, there was significant difference ($P<0.05$) in both groups with the course of infection, i.e. at day 23 PI the cattle showed a reduced number of red blood cells, hematocrit and hemoglobina concentration. MCV and MCHC values did not differ significantly between animals and groups ($P>0.05$). Fibrinogen was upper in splenectomized than intact on day 0 ($P>0.05$). The total plasma protein levels did not differ between groups and did not change with the evolution of infection ($P>0.05$).

Leucogram data were presented in the Table 2. There was no difference between the two groups ($p>0.05$) for the number of leukocytes throughout the experiment. But an increase of the total number of leukocytes in both groups (intact and splenectomized) after 15 days of infection ($P <0.05$) was observed (Table 2). This increase in total leukocytes is related to the increase of lymphocytes in infected animals (both groups). In the number of neutrophils, monocytes and eosinophils there was no significant difference between groups and in the same group ($p> 0.05$).

3.5 Oxidative profile and antioxidant status

Results of TBARS and NPSH are shown in Table 3, and show that the levels of these two variables did not differ between groups, that is, the same alterations occurred in intact or splenectomy animals. But when was analyzed the evolution of the disease, we found that there was a significant increase in TBARS levels at days 21 and 23 PI in splenectomized and at day 23 in intact group, and in that period there was a reduction in the NPSH levels ($P<0.05$) in animals of both groups compared on day 0 of the experiment.

Correlation analyzes were performed (Figure 2 and 3). Thus positive correlation was observed ($P<0.05$) between bacteremia and TBARS levels in intact animals ($R^2=0.52$) and splenectomized ($R^2=0.54$). In contrast, a negative correlation ($P<0.05$) was observed between bacteremia and NPSH levels in intact animals ($R^2= -0.44$) and splenectomized ($R^2= -0.66$). However, when correlated NPSH and TBARS a negative correlation was observed in both groups, i.e., intact ($R^2= -0.53$) and splenectomized ($R^2= -0.52$), indicating an oxidative imbalance.

4. Discussion

Hematological parameters were performed to evaluate the course of the disease, and show that the infection caused alterations compatible with bovine anaplasmosis. Hematologic results of our study were similar to those reported by Nazifi et al. (2012) in a study of cattle naturally infected by *A. marginale*, which showed a significant decrease in red blood cell count, hematocrit and hemoglobina. The animals of this study developed a mild normocytic normochromic anemia. These findings are due to common extravascular erytrophagocytosis bovine anaplasmosis (Ristic, 1981), it is noteworthy that in the acute infection anemia is normocytic and later evolves into macrocytic, with bone marrow hyperplasia, reticulocytosis, increased MCV and osmotic fragility of red blood cells (Thrall, 2012). The elevation of fibrinogen in the experiment beginning in splenectomized cattle (Day 0) results still tissue repair due to surgical recovery (Feldman et al., 2000), unrelated to infection by *A. marginale*.

According to literature, the total leukocytes counts in cattle may show little variation, even during acute inflammation (Thrall, 2012). In this study an increase of total leukocytes occurred as a result of lymphocytosis, this cell increased in the circulation in order to combat

the etiological agent and form an immune response, findings described in other studies in bovine anaplasmosis (Alsaad and Alimam, 2013).

A negative correlation between TBARS and NPSH levels in both groups, which characterizes a situation of lipid peroxidation without a non-enzymatic antioxidant response, similar to those described in cattle infected with *Babesia bovis* (Commins et al., 1988). This data indicates that infected animals are having cell and/or tissue lesions, and the antioxidant barriers (NPSH) is not responding to oxidative increase and also reduced in these animals, this being interpreted as a negative effect on the animal.

In our study there was an increase in TBARS levels with increasing bacteremia in all infected animals, similar to results for De et al. (2012) in erythrocytes of cattle infected by *A. marginale*, which indicates osmotic fragility and may contribute to the pathogenesis of anemia, characteristic of anaplasmosis. Independent group (with or without spleen), infected cattle have lipid peroxidation in serum, which may be occurring due to hemolysis caused by the etiological agent. Similar correlation was also observed in other hemoparasitoses, as in cattle naturally infected by *Babesia bigemina* (Saleh, 2009), where the animals showed an increase in serum MDA positively associated with parasitaemia. The increase in MDA levels was also described in cattle naturally infected by *A. marginale* (Ergoenuel and Askar, 2009) and by *Theileria annulata* (Asri Rezaei and Dalir-Naghadeh, 2006). Oxidative stress studies are also described in sheep naturally infected by *Babesia ovis* (Esmaeilnejad et al., 2012), in horses naturally infected with *Theileria equi* (Cingi et al., 2012) and dogs naturally infected by *Babesia gibsoni* (Chaudhuri et al., 2008) and *Babesia canis* (Crnogaj et al., 2010).

In this study, the NPSH levels reduced in erythrocytes of animals infected by *A. marginale*, regardless of the presence or absence of spleen. Note that the NPSH levels corresponds to the non enzymatic antioxidants, and in *B. bovis* infection (also a parasite erythrocytes) was observed a decrease in the antioxidant vitamin E and in sialic acid

activities (Commins et al., 1988). Cattle naturally infected with *A. marginale* had reduced superoxide dismutase and glutathione activities in the blood (More et al., 1989; Nazifi et al., 2012; Nazifi et al., 2008). However, the enzymatic antioxidant such as catalase increases in erythrocytes extract infected by *A. marginale*, which is directly proportional increase in the activity level of bacteremia (Wallace and Dimopoullos, 1965). A recent study investigated superoxide dismutase activity, glutathione peroxidase, catalase and in sheep experimentally infected with *Anaplasma ovis*, and found that the three enzymes has its activity increased on acute infection, but with the evolution of the disease glutathione peroxidase activity decreased in erythrocytes (Yasini et al., 2014). Based on our results and the literature, we found that antioxidant status reduces during infection by *A. margnale* in cattle, which is not good for the host, as well does not occur the hydrolysis of ROS, which are toxic to cells and tissues.

Based on the results it is possible to conclude that *A. marginale* infection in cattle causes oxidative and antioxidant imbalance in these animals, regardless of whether or not the spleen. The high bacteremia is directly related to lipid peroxidation, as well as related to the reduction of non-enzymatic antioxidant.

Table 1: Medians and maximum and minimum values of sequential haematological analysis of cattle experimentally infected by *Anaplasma marginale*.

		Day 0	Day 8	Day 15	Day 21	Day 23
		Total erythrocyte (x10^6/µL)				
Intact	median	9.70 ^{1ab}	9.70 ^{1a}	9.64 ^{1ab}	9.14 ^{1ab}	8.05 ^{1b}
	max-min	(11.1-9.1)	(11.4-9.6)	(10.5-9.2)	(9.9-8.4)	(9.5-6.5)
Splenect.	median	9.20 ^{1a}	9.21 ^{1ab}	8.31 ^{1ab}	8.91 ^{1ab}	8.23 ^{1b}
	max-min	(10.5-8.9)	(10.1-7.4)	(10.1-7.6)	(10.9-8.6)	(6.1-9.6)
		Hemoglobin (g/dL)				
Intact	median	11.85 ^{1a}	11.60 ^{1a}	10.90 ^{1a}	10.15 ^{1ab}	9.30 ^{1b}
	max-min	(12.0-9.8)	(12.2-11.0)	(11.7-10.7)	(11.3-9.4)	(10.4-7.8)
Splenect.	median	10.15 ^{1a}	10.0 ^{1ab}	9.30 ^{1ab}	9.60 ^{1a}	9.25 ^{1b}
	max-min	(11.3-9.8)	(11.0-8.7)	(10.8-8.2)	(11.7-9.2)	(10.4-7.0)
		Hematocrit (%)				
Intact	median	38.5 ^{1a}	36.5 ^{1a}	36.5 ^{1a}	30.5 ^{1ab}	28.0 ^{1b}
	max-min	(40-31)	(39-34)	(39-34)	(35-28)	(31-24)
Splenect.	median	34.0 ^{1a}	31.5 ^{1a}	29.5 ^{1a}	29.5 ^{1ab}	28.5 ^{1b}
	max-min	(37-32)	(36-27)	(33-27)	(34-29)	(31-21)
		MCV (fL)				
Intact	median	37.15 ^{1a}	36.90 ^{1a}	36.95 ^{1a}	37.35 ^{1a}	37.85 ^{1a}
	max-min	(42.8-34.3)	(41.5-34.7)	(41.7-34.2)	(42.0-35.0)	(42.9-34.8)
Splenect.	median	35.95 ^{1a}	35.40 ^{1a}	35.8 ^{1a}	36.3 ^{1a}	37.15 ^{1a}
	max-min	(37.3-35.5)	(36.2-34.9)	(36.6-34.9)	(37.2-34.7)	(38.9-35.4)
		MCHC (%)				
Intact	median	30.78 ^{1a}	31.78 ^{1a}	30.05 ^{1a}	33.15 ^{1a}	33.17 ^{1a}
	max-min	(31.6-30.0)	(32.3-31.3)	(31.4-29.9)	(33.9-32.3)	(33.5-32.5)
Splenect.	median	30.42 ^{1a}	31.73 ^{1a}	30.90 ^{1a}	33.1 ^{1a}	33.33 ^{1a}
	max-min	(30.6-29.4)	(32.2-30.6)	(31.7-29.1)	(34.4-30.7)	(33.5-31.7)
		Plasma proteins (g/dL)				
Intact	median	8.0 ^{1a}	8.5 ^{1a}	8.2 ^{1a}	8.0 ^{1a}	8.0 ^{1a}
	max-min	(8.4-7.6)	(8.8-8.0)	(8.4-8.0)	(8.4-7.8)	(8.2-7.8)
Splenect.	median	7.8 ^{1a}	7.9 ^{1a}	7.9 ^{1a}	8.1 ^{1a}	7.9 ^{1a}
	max-min	(8.2-7.4)	(8.0-7.6)	(8.6-7.4)	(8.6-7.4)	(8.2-7.0)
		Fibrinogen (mg/dL)				
Intact	median	300 ^{1a}	300 ^{1a}	400 ^{1a}	400 ^{1a}	400 ^{1a}
	max-min	(400-200)	(400-200)	(400-200)	(600-400)	(600-200)
Splenect.	median	600 ^{2a}	400 ^{1a}	500 ^{1a}	400 ^{1a}	400 ^{1a}
	max-min	(600-600)	(600-200)	(600-400)	(400-400)	(600-200)

NOTE: Subscript numbers (^{1} or ^{2}) give significant differences between the intact animals and splenectomy in a single study period (columns). Letters (^{a} or ^{b}) confer significant differences in intact or splenectomy groups during different days evaluated (lines).

Table 2: Medians and maximum and minimum values of sequential leucogram of cattle experimentally infected by *Anaplasma marginale*.

		Day 0	Day 8	Day 15	Day 21	Day 23
Total leukocyte (/µL)						
Intact	median	8400 [1b]	9100 [1ab]	9650 [1ab]	8250 [1b]	12300 [1a]
	max-min	(9400-7800)	(1300-8200)	(11500-6600)	(8600-7900)	(13700-7600)
Splenect.	median	9350 [1a]	8500 [1a]	12900 [1b]	9400 [1ab]	10500 [1ab]
	max-min	(10900-7200)	(10600-4400)	(14000-10500)	(9600-8100)	(12000-9000)
Neutrophils (µL)						
Intact	median	2177 [1a]	1905 [1a]	1910 [1a]	2440 [1a]	2929 [1a]
	max-min	(3120-1445)	(5460-1476)	(3888-1254)	(2923-2158)	(4420-1276)
Splenect.	median	3412 [1a]	1871 [1a]	4820 [1a]	2865 [1a]	3630 [1a]
	max-min	(3597-2664)	(4558-869)	(4900-4725)	(3648-2280)	(5390-2900)
Lymphocytes (µL)						
Intact	median	6082 [1a]	6479 [1ab]	6073 [1ab]	5794 [1a]	8540 [1b]
	max-min	(6800-4056)	(8316-5312)	(8855-5280)	(5893-4898)	(9316-5776)
Splenect.	median	5216 [1a]	4926 [1a]	6677 [1b]	6097 [1ab]	6700 [1ab]
	max-min	(6758-4104)	(6399-3168)	(8960-5250)	(7125-5103)	(6700-5280)
Monocytes (µL)						
Intact	median	222 [1a]	495 [1a]	206 [1a]	84 [1a]	369 [1a]
	max-min	(830-156)	(520-0)	(864-0)	(249-79)	(650-76)
Splenect.	median	453 [1a]	601 [1a]	280 [1a]	41 [1b]	365 [1a]
	max-min	(846-186)	(848-132)	(976-0)	(95-0)	(1080-0)
Eosinophils (µL)						
Intact	median	83 [1a]	91 [1a]	97 [1a]	0 [1a]	65 [1a]
	max-min	(390-0)	(166-0)	(690-0)	(164-0)	(580-0)
Splenect.	median	36 [1a]	135 [1a]	121 [1a]	129 [1a]	0 [1a]
	max-min	(93-0)	(212-79)	(244-0)	(186-0)	(240-0)

NOTE: Subscript numbers ([1] or [2]) give significant differences between the intact animals and splenectomy in a single study period (columns). Letters ([a] or [b]) confer significant differences in intact or splenectomy groups during different days evaluated (lines).

Table 3: Medians and maximum and minimum values of oxidative markers (TBARS) and antioxidant (NPSH) of cattle experimentally infected by *Anaplasma marginale*.

		Day 0	**Day 8**	**Day 15**	**Day 21**	**Day 23**
		TBARS levels (nmol MDA/mL)				
Intact	median	30.81 [1a]	29.57 [1a]	30.70 [1a]	86.20 [1a]	111.87 [1b]
	max-min	(34.29-20.13)	(32.19-26.43)	(35.76-29.20)	(133.75-33.53)	(156.64-44.42)
Splenec.	median	34.29 [1a]	30.60 [1a]	32.86 [1a]	79.70 [1b]	157.80 [1b]
	max-min	(54.63-24.97)	(42.63-29.87)	(50.03-31.29)	(280.87-56.19)	(324.45-75.98)
		NPSH levels (µmol/ml)				
Intact	median	1.80 [1a]	1.60 [1a]	1.45 [1ab]	1.34 [1b]	1.35 [1b]
	max-min	(1.93-1.68)	(1.73-1.49)	(1.53-1.38)	(1.50-1.27)	(1.71-0.97)
Splenec.	median	1.97 [1a]	1.92 [1a]	1.76 [1ab]	1.44 [1b]	1.21 [1b]
	max-min	(2.10-1.81)	(1.92-1.80)	(1.91-1.57)	(1.87-0.87)	(1.62-0.66)

NOTE: Subscript numbers ([1] or [2]) give significant differences between the intact animals and splenectomy in a single study period (columns). Letters ([a] or [b]) confer significant differences in intact or splenectomy groups during different days evaluated (lines).

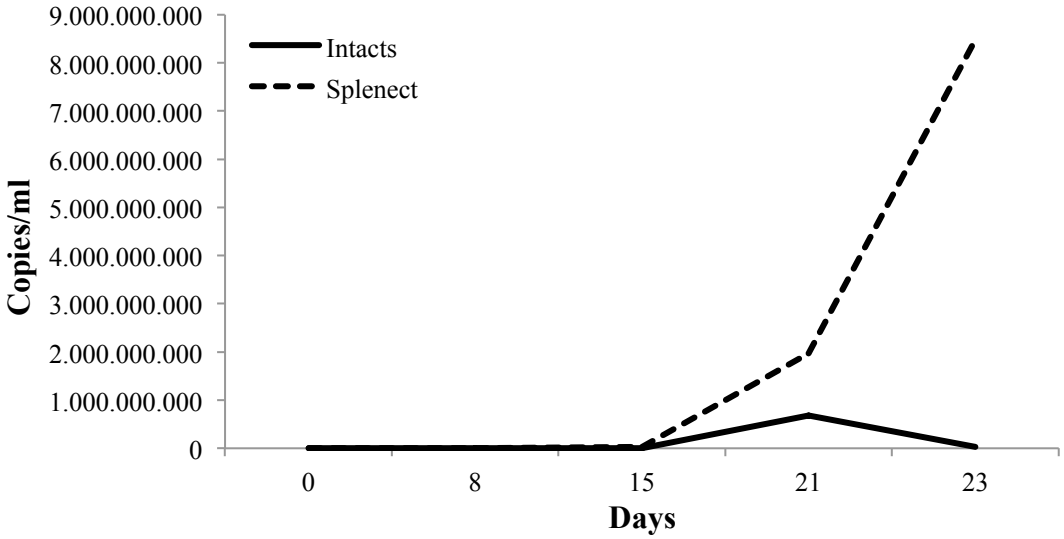

Figure 1. Progression of bacteremia (copies/ml) in whole blood in intact and splenectomized cattle experimentally infected by *Anaplasma marginale*.

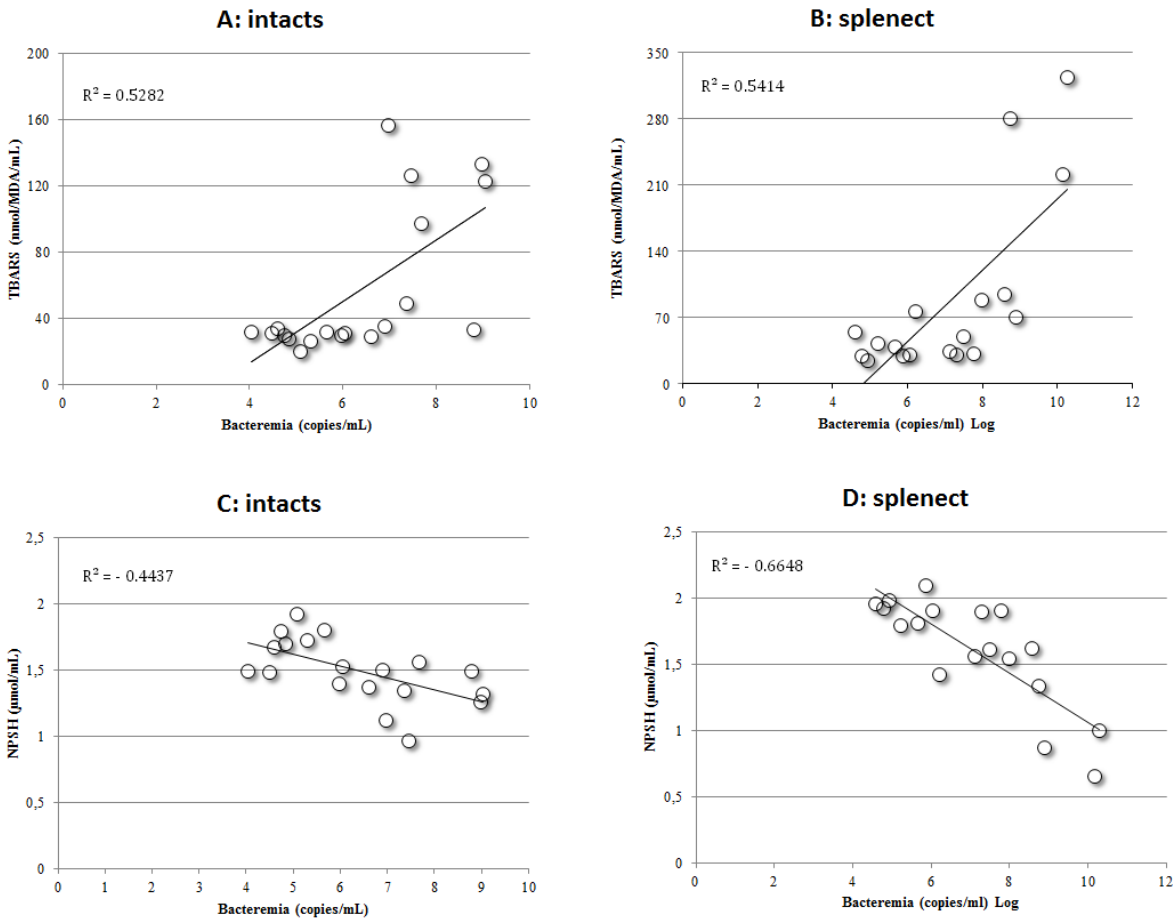

Figure 2: Analysis of correlation between bacteremia and TBARS, as well as between NPSH and bacteremia in intact (A, C) and splenectomized animals (B, D), and infected by *A. marginale* (P<0.05).

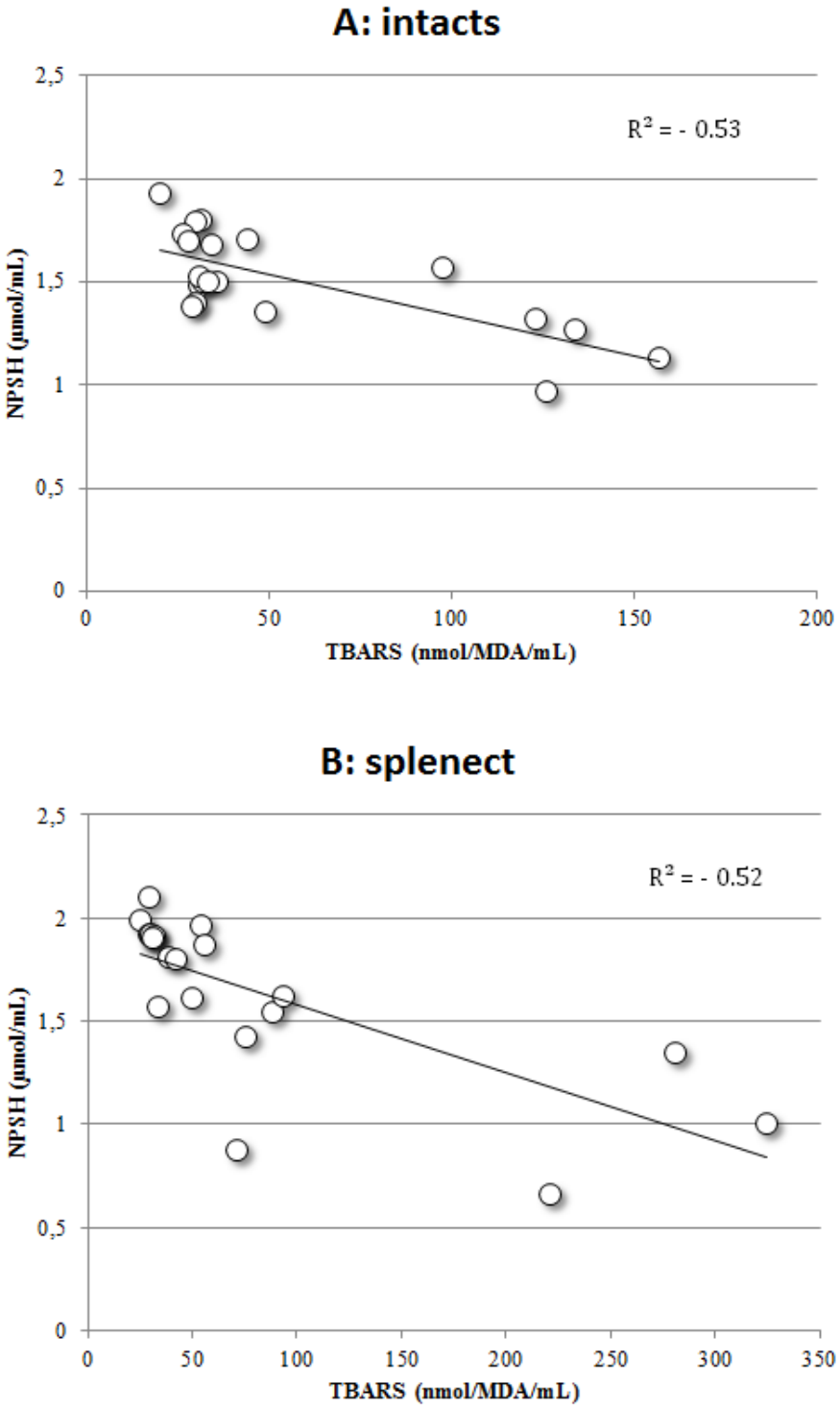

Figure 3: Analysis of correlation between TBARS and NPSH in intact (A) and splenectomized animals (B), and infected by *A. marginale* (P<0.05).

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5. CONSIDERAÇÕES FINAIS

A avaliação do perfil oxidativo e as alterações inflamatórias em infecções isoladas dos três principais agentes da Tristeza Parasitária Bovina (TPB) no Brasil, auxilou no esclarescimento dos mecanismos envolvidos principalmente na lesão eritrocitária causada por estes parasitas.

Em relação à infecção experimental com cepa atenuada de *B. bovis* foi observada parasitemia muito baixa. Entretanto, mesmo sem manifestações clinicas, os animais apresentaram alterações hematológicas significativas, como o decréscimo na contagem de hemácias, porém não sendo caracterizado o quadro de anemia característico da infecção natural por este parasita (Yokoyama et al. 2006; Saleh 2009). Embora a diminuição na contagem de hemácias não tenha ultrapassado os valores de referência para a espécie (Thrall, 2012), foi suficiente para correlacionar negativamente com o aumento nos níveis de susbtâncias reativas ao acido tiobarbitúrico (TBARS), um indicador indireto de peroxidação lipídica. Como forma de proteção às lesões oxidativas causadas pelo excesso de espécies reativas de oxigênio (ERO), o organismo consome suas reservas antioxidantes, comprovado pelo decréscimo na atividade das enzimas catalase (CAT) e superóxido dismutase (SOD).

Também foram investigados neste estudo, bovinos infectados por cepa atenuada de *B. bigemina*, os quais apresentaram baixa parasitemia concomitante com decréscimo nos valores de hemoglobina e hematócrito, característicos da destruição eritrocitária ocorrida pela multiplicação do parasita (Alkhalil et al., 2007; Ristic, 1981). Outro importante dado observado nesta infecção experimental, foi a diminuição na atividade das colinesterases sanguíneas (AChE e BChE). Pode-se inferior que a perda da integridade da membrana eritrocitária pode estar relacionada com este resultado, visto que estas enzimas estão ancoradas na membrana da célula. Outro importante dado observado neste estudo é em relação à peroxidação lipídica no soro dos animais infectados, o que pode corroborar na interpretação destes resultados.

Por outro lado, estas colinesterases sanguíneas atuam como marcadores inflamatórios de baixo grau (Das, 2012), Com o decréscimo na atividade destas enzimas, pode-se sugerir um aumento do neurotransmissor Ach no meio extracellular, inibindo a liberação de interleucina-6 e fator de necrose tumoral-α, assim como outros agentes pró-inflamatórios pelos linfócitos, o que torna as atividades destas enzimas importantes reguladoras intrínsecas da inflamação (Anglister et al., 2008; Das, 2007).

Ainda neste estudo com a cepa atenuada de *B. bigemina*, foi observada uma diminuição da atividade da CAT e aumento da atividade da SOD, devido a mecanismos regulatorios endógenos dispendidos na tentativa de neutralizar os efeitos deletérios da peroxidação lipídica.

Em relação à infecção de bovinos esplenectomizados e intactos com cepa virulenta de *A. marginale*, foi observado um quadro hematológico característico de hemólise extravascular descrito na anaplasmose (Ristic, 1981). Sendo a anemia hemolítica mais acentuada nos animais esplenectomizados devido à elevação da bacteremia causada pela não remoção das hemácias parasitadas pelos macrófagos esplênicos. A progressão da bacteremia apresentou correlação positiva com o aumento nos níveis de TBARS e negativa com o conteúdo de tióis não protéicos (NPSH), caracterizando um quadro de peroxidação lipídica.

Baseado nestes resultados pôde-se verificar que as alterações oxidativas e inflamatórias são detectadas muito antes do aparecimento das manifestações clínicas características da Tristeza Parasitária Bovina, mesmo sob baixa parasitemia. No caso da infecção por *B. bovis*, em que o período de incubação è de até 12 dias, foi possível detectar alterações oxidativas a partir do dia 7 pós-infecção.

No experimento realizado com uma cepa atenuada de *B. bigemina* foi possível produzir uma infecção assintomática por 15 dias, estendendo o período de incubação de uma cepa virulenta (6-8 dias), sendo possível detectar alterações significativas no perfil oxidativo e inflamatório a partir do dia 7 PI.

Na infecção de bovinos esplenectomizados e intactos com *A. marginale*, pudemos verificar que as alterações oxidativas são decorrentes do aumento da bacteremia e esta então, é intensificada pela ausência na remoção das hemácias parasitadas pelos macrófagos esplênicos principalmente.

Neste estudo pode-se concluir que a TPB é capaz de desencadear um quadro de estresse oxidativo demonstrado pelo aumento da peroxidação lipídica e consumo dos mecanismos antioxidantes evidenciados nas infecções experimentais com os três agentes isolados. Em adição pode-se sugerir que a avaliação de parâmetros de estresse oxidativo e de marcadores inflamatórios de baixo grau podem ser utilizados como base para futuros estudos referentes ao diagnostico precoce desta enfermidade assim como o uso de antioxidantes na alimentação animal, a fim de reduzir a interferência do estresse oxidativo na patogênese da TPB.

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