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BRUCELOSE NATURAL E EXPERIMENTAL: DISTÚRBIOS BIOQUÍMICOS ENVOLVIDOS NA PATOGENIA DA DOENÇA

GÉSSICA PERIN

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BRUCELOSE NATURAL E EXPERIMENTAL: DISTÚRBIOS BIOQUÍMICOS ENVOLVIDOS NA PATOGENIA DA DOENÇA

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BRUCELOSE NATURAL E EXPERIMENTAL: DISTÚRBIOS BIOQUÍMICOS ENVOLVIDOS NA PATOGENIA DA DOENÇA

Elaborada por
Géssica Perin

como requisito parcial para obtenção do grau de
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RESUMO

Dissertação de Mestrado
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A brucelose é uma doença infecciosa causada por bactérias do gênero *Brucella*. Apresenta ampla distribuição mundial, sendo considerada uma das principais causas de aborto, problemas reprodutivos e descarte precoce dos animais, além de prejuízos para a produção de bovinos e ovinos devido às significativas perdas econômicas e barreiras sanitárias. Sabe-se que entre os mecanismos fisiológicos, a formação de radicais livres ocorre naturalmente, no entanto, sob condições patológicas, como na brucelose, pode haver uma superprodução desses radicais, causando danos às células e aos tecidos e, portanto, exigindo uma maior ativação do sistema antioxidante, do que em situações normais. Nestas mesmas condições, é enfatizada a importância de estudos sobre as enzimas que atuam como marcadores inflamatórios, como é o caso da adenosina deaminase (ADA), colinesterases e ectonucleotidases. Portanto, o objetivo deste estudo foi avaliar se há ocorrência de estresse oxidativo e alteração na atividade de enzimas purinérgicas, colinérgicas e do metabolismo energético em animais infectados para *Brucella abortus* e *Brucella ovis* em condição natural e experimental, respectivamente, além de avaliar a soroprevalência de *B. abortus* em propriedades localizadas no oeste do estado de Santa Catarina. Para atingir os objetivos, três experimentos foram conduzidos. Para o experimento I, foram selecionadas amostras de sangue e soro de vacas de um rebanho do oeste do estado de Santa Catarina, alimentados com a mesma dieta, e com suspeita de brucelose por apresentarem histórico de aborto no último terço de gestação e repetições estro constantes. Entre esses animais, foram selecionados o soro de 10 animais positivos para *B. abortus* e 10 negativos. Observou-se nos animais soropositivos uma redução na atividade da ADA, assim como da enzima catalase (CAT). Já a peroxidação lipídica (TBARS) e atividade da superóxido dismutase (SOD) foi maior nas vacas soropositivas ($P<0,05$). Portanto conclui-se que as vacas estão em condição de estresse oxidativo e uma redução da ADA visa diminuir a resposta inflamatória, visto que essa enzima é responsável por regular níveis da molécula anti-inflamatória conhecida como adenosina. O experimento II foi conduzido em propriedades do oeste do estado que não estão em vigilância para a doença brucelose. Um total de 1242 amostras de soro oriundas de vacas da região oeste de Santa Catarina foram submetidas ao protocolo sorodiagnóstico conhecido como teste de Antígeno Acidificado (AAT) e em seguida ao teste de 2-Mercaptoetanol (2-ME) para fins de confirmação. Todas as amostras avaliadas foram soronegativas para *B. abortus*. Embora não tenhamos encontrado animais soropositivos para brucelose em nosso estudo, a doença ainda requer uma vigilância contínua, devido ao seu impacto econômico e ao estresse oxidativo causado por ela, o que pode ter contribuído para casos de aborto em três vacas soropositivas (Experimento I) no terço final da gestação após analises bioquímicas. No experimento III utilizou-se 48 camundongos, sendo que 50% deles foram infectados experimentalmente por *B. ovis* e os demais foram utilizados como controle (não infectados). Parâmetros hematológicos, bioquímicos e histopatológicos foram avaliados nesses animais em quatro momentos (dia 7, 15, 30 e 60 pós-infecção (PI)). Observou-se que a infecção pela bactéria *B. ovis* em modelo experimental provoca distúrbios hematológicos como uma redução dos níveis de hematócrito

e aumento da contagem de leucóticos; alterações bioquímicas como diminuição dos níveis séricos de albumina e aumento de globulinas (dia 15, 30 e 60 PI), e patológicos como esplenomegalia, esplenite granulomatosa e granulomas hepáticos multifocais. A redução dos níveis de hematócrito na fase aguda da infecção, bem como a resposta inflamatória exacerbada, pode contribuir para danos no tecido esplênico e hepático, e assim pode alterar a função hepática, diminuindo os níveis de albumina. A atividade da AChE foi elevada em sangue e homogenizado de cérebro, bem como a BChE em soro. Este aumento é caracterizado como uma resposta pró-inflamatória, visto que, estas enzimas regulam os níveis de acetilcolina (ACh), a qual apresenta efeito anti-inflamatório. Além disso, a infecção por *B. ovis* reduziu a atividade das enzimas NTPDase e 5'-nucleotidase em cérebro na fase aguda, com posterior elevação na fase crônica, sugerindo que a hidrólise de nucleotídeos é baixa na fase aguda devido à diminuição destas enzimas. O mesmo ocorreu com a ADA em soro, enzima responsável por regular os níveis de adenosina extracelular. A diminuição de sua atividade na fase aguda sugere ação anti-inflamatória, ao passo que, com a cronificação da doença, eleva sua atividade na tentativa de combater o agente, apresentando então ação pró-inflamatória. A resposta inflamatória também é evidenciada pelo aumento dos níveis de proteína C-reativa em soro nos animais infectados, visto que, esta proteína é produzida pelo fígado em resposta a estímulos inflamatórios, infecção e dano tecidual. Quanto as enzimas do metabolismo energético, observou-se nos animais infectados uma elevação da atividade cardíaca da creatina quinase (CK) citosólica (30 e 60 PI) e diminuição da creatina quinase mitocondrial (30 e 60 PI) bem como da piruvato quinase (PK) (60 PI). Estas alterações sugerem um comprometimento da síntese e regulação de ATP, associado a um aumento na produção de espécies reativas ao oxigênio (ROS) em soro, baço, fígado, coração, cérebro e nas concentrações de TBARS cerebrais e cardíacos observados em nosso estudo. A ativação dos mecanismos antioxidantes foi evidenciada pelo aumento dos níveis séricos e cardíacos de SOD, bem como a redução das concentrações séricas de CAT e de níveis de tióis não-proteicos (NPSH) em coração. Portanto concluímos que infecções por *B. abortus* e *B. ovis* geram um quadro de estresse oxidativo a nível sérico e tecidual, que pode contribuir negativamente para a doença, assim como concluímos também que enzimas dos sistemas purinérgico, colinérgico e metabolismo energético participam da regulação da resposta inflamatória da doença de forma direta ou indireta, conforme detalhado nessa dissertação.

Palavras-chave: brucelose, colinesterases, danos celulares, estresse oxidativo, metabolismo energético, sistema purinérgico.

ABSTRACT
Master's Dissertation
Programa de Pós-Graduação em Zootecnia
Universidade do Estado de Santa Catarina

NATURAL AND EXPERIMENTAL BRUCELLOSIS: BIOCHEMICAL DISORDERS INVOLVED IN PATHOGENESIS OF DISEASE

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Chapecó, August 22, 2018

Brucellosis is an infectious disease caused by bacteria of the genus *Brucella.* This file was distributed by the worldwide company, being one of the main causes of risk, reproductive problems and early discontinuity of the animals, besides losses for cattle and sheep production in the last decades and sanitary barriers. It is known that among the physiological mechanisms, the formation of free radicals occurs naturally, however, under pathological conditions, as in brucellosis, there may be an overproduction of these radicals, causing damage to cells and tissues and, therefore, requiring a greater activation of the antioxidant system than in normal situations. Under these same conditions, the importance of studies on the enzymes that act as inflammatory markers, such as adenosine deaminase (ADA), cholinesterases and ectonucleotidases, is emphasized. Therefore, the objective of this study was to evaluate the occurrence of oxidative stress and alteration in the activity of purinergic, cholinergic and energetic metabolism in infected animals for *Brucella abortus* and *Brucella ovis* in natural and experimental conditions, respectively, besides evaluating the seroprevalence of *B. abortus* on properties located in the west of the state of Santa Catarina. To achieve the objectives, three experiments were conducted. For the experiment I, blood and serum samples of cows from a herd of the state of Santa Catarina, fed with the same diet, and with suspected brucellosis were selected because they presented a history of abortion in the last third of gestation and repetitions of estrus. Among these animals, 10 sera were positive for *B. abortus* and 10 were negative. In the seropositive animals a reduction in ADA activity was observed, as well as of the enzyme catalase (CAT). On the other hand, lipid peroxidation (TBARS) and superoxide dismutase activity (SOD) were higher in seropositive cows ($P < 0.05$). Therefore it is concluded that cows are in a condition of oxidative stress and a reduction of ADA aims to decrease the inflammatory response, since this enzyme is responsible for regulating levels of the anti-inflammatory molecule known as adenosine. Experiment II was conducted on western properties of the state that are not under surveillance for the brucellosis disease. A total of 1242 serum samples from cows from the western region of Santa Catarina were submitted to the serodiagnostic protocol known as the Acidified Antigen Test (AAT) and then to the 2-Mercaptoethanol (2-ME) test for confirmation purposes. All samples tested were seronegative to *B. abortus*. Although we did not find seropositive animals for brucellosis in our study, the disease still requires continuous surveillance due to its economic impact and the oxidative stress caused by it, which may have contributed to abortion cases in three seropositive cows (Experiment I) in the final third of gestation after biochemical analysis. In the experiment III, 48 mice were used, of which 50% were experimentally infected by *B. ovis* and the others were used as control (non-infected). Hematological, biochemical and histopathological parameters were evaluated in these animals at four times (days 7, 15, 30 and 60 post-infection (PI)). It was observed that infection by the *B. ovis* bacterium in the experimental model causes hematological disorders such as a reduction in hematocrit levels and an increase in leukocyte count; biochemical changes such

as decreased serum albumin levels and increased globulins (day 15, 30 and 60 PI), and pathological conditions such as splenomegaly, granulomatous splenitis and multifocal hepatic granulomas. Reduced hematocrit levels in the acute phase of infection, as well as the exacerbated inflammatory response, may contribute to splenic and hepatic tissue damage, and thus may alter liver function, decreasing albumin levels. AChE activity was elevated in blood and brain homogenate, as well as serum BChE. This increase is characterized as a pro-inflammatory response, since these enzymes regulate the levels of acetylcholine (ACh), which has an anti-inflammatory effect. In addition, *B. ovis* infection reduced the activity of the NTPDase and 5'-nucleotidase enzymes in the acute phase, with subsequent elevation in the chronic phase, suggesting that the nucleotide hydrolysis is low in the acute phase due to the decrease of these enzymes. The same occurred with ADA in serum, an enzyme responsible for regulating extracellular adenosine levels. The reduction of its activity in the acute phase suggests an anti-inflammatory action, whereas, with the chronification of the disease, it raises its activity in the attempt to combat the agent, presenting then pro-inflammatory action. The inflammatory response is also evidenced by increased levels of C-reactive protein in serum in infected animals, since this protein is produced by the liver in response to inflammatory stimuli, infection and tissue damage. As for energetic metabolism enzymes, an increase in cytosolic creatine kinase (CK) and cytosolic creatine kinase activity (30 and 60 PI) and decrease in mitochondrial creatine kinase (30 and 60 PI) as well as pyruvate kinase (PK) (60 PI). These alterations suggest a compromise in the synthesis and regulation of ATP, associated to an increase in the production of oxygen reactive species (ROS) in serum, spleen, liver, heart, brain and brain and cardiac TBARS concentrations observed in our study. Activation of the antioxidant mechanisms was evidenced by increased serum and cardiac levels of SOD, as well as the reduction of serum concentrations of CAT and non-protein thiol levels (NPSH) in the heart. Therefore, we conclude that *B. abortus* and *B. ovis* infections generate a picture of oxidative stress at the serum and tissue levels, which may contribute negatively to the disease, and also conclude that purinergic, cholinergic and energy metabolism enzymes participate in the regulation of inflammatory response of the disease directly or indirectly, as detailed in this dissertation.

Keywords: brucellosis, cellular damage, purinergic system, cholinesterases, oxidative stress, energy metabolism.

SUMÁRIO

1. CAPÍTULO I

REVISÃO DE LITERATURA

1.1 BRUCELOSE

A brucelose é uma doença infectocontagiosa crônica, que acomete diferentes espécies animais. As bactérias do gênero *Brucella* são gram-negativas, intracelulares facultativas, aeróbias ou microaerófilas, imóveis, não esporulam e não encapsulam (CORBEL & MORGAN, 1984). Apresentam-se morfologicamente como cocobacilos curtos de 0,6 a 1,5 µm por 0,5 a 0,7 µm (PROBERT et al., 2004) e multiplicam-se entre 20 a 40°C, em meio rico, sendo 37°C a temperatura e um pH de 6,6 a 7,4 ideal (OIE, 2009).

Nove espécies são reconhecidas dentro do genêro *Brucella*, cada qual com seus hospedeiros preferenciais, sendo consideradas clássicas a *B. melitensis* (caprinos, ovinos e camelos), *B. abortus* (bovinos e bubalinos), *B. suis* (suínos e javalis), *B. neotomae* (ratos do deserto), *B. ovis* (ovelhas) e *B. canis* (cães). Novas espécies estão sendo estudadas, isoladas de mamíferos marinhos (WHATMORE et al, 2017). Com exceção da *B. ovis* e *B. neotomae* as demais espécies clássicas apresentam potencial zoonótico (TILLER et al., 2010). Devido características de multiplicação e a constituição de sua parede celular, as superfícies destas bactérias podem ser classificadas como lisas ou rugosas, devido à presença ou ausência da cadeia O. A cadeia O compõe o lipopolissacáride (LPS), relacionado à virulência das espécies e que se encontra externamente na superfície da *Brucella* spp. (NIELSEN et al. 2004; CARDOSO et al., 2006). O LPS é constituído pelo lipídeo A, o núcleo oligossacáride e a cadeia O nas colônias lisas (*B. melitensis, B. abortus, B. suis e B. neotomae*) enquanto que, as rugosas (*B. canis e B. ovis*) apresentam somente o lipídeo A e o núcleo oligossacáride (PAULIN & FERREIRA NETO, 2003; LAGE et al., 2008a).

A *Brucella* spp. apresenta capacidade de proliferação intracelular em células fagocíticas como macrófagos e em fagócitos como trofoblasto (THOEN et al., 1993; SILVA et al., 2005). Para a sobrevivência do microorganismo no hospedeiro é fator determinante sua capacidade de alterar a maturação do fagossomo, escapando da fusão com o lisossomo e da degradação bacteriana, o que possibilita alcançar seu local de replicação intracelular, resultando em infecção crônica e persistente (GORVEL E MORENO, 2002).

Estas bactérias têm como características ocasionar alterações reprodutivas, resultando em perdas produtivas importantes nas espécies domésticas (SILVA et al., 2005) enquanto que, na espécie humana, os sinais clínicos são inespecíficos, como febre,

emagrecimento, depressão, artrite e, menos frequentemente, problemas reprodutivos (KO & SPLITTER, 2003). Com distribuição mundial, a brucelose é considerada como um grave problema ligado à saúde pública (BRASIL, 2006), sendo apontada como a zoonose bacteriana maior prepoderância em todo o mundo, com notificação de mais de 500.000 novos casos anuais de infecção humama (FRANCO et al., 2007).

O primeiro caso descrito no homem foi em 1959, com sinais como febre ondulante com consequente morte, na Ilha de Malta, no Mar Mediterrâneo, sendo por isso nomeada Febre de Malta (POESTER et al., 2009). Em 1895 identificou-se como *Bacillus abortus* em útero e membranas fetais abortadas de vacas (POESTER et al., 2009). No Brasil o primeiro em 1913, e, em 1914 foi realizado o primeiro diagnóstico da brucelose bovina no país, no estado do Rio Grande do Sul (VIEIRA, 2004).

1.1.1 *Brucella abortus*

A *B. abortus* (biovariedades 1-6,9) acomete bovinos e bubalinos, além de ter caráter zoonótico. No Brasil, em função de áreas com elevadas prevalências em alguns estados (até 10% do rebanho), assim como bloqueios sanitários da fazenda e dos rebanhos, a brucelose torna-se responsável por enormes prejuízos à bovinocultura. A patogenicidade desta bactéria relaciona-se a mecanismos que concedem sua invasão, sobrevivência e multiplicação no interior das células do hospedeiro, preservando-se da ação do sistema imune (XAVIER et al., 2009).

Apresenta como porta de entrada principal a mucosa orofaringeana (RIBEIRO et al., 2008), e, posteriormente são fagocitadas, processo realizado especialmente por macrófagos e então conduzidas até os linfonodos regionais, para multiplicação e onde podem se manter por longos períodos, processo pelo qual acarreta em hiperplasia e linfadenite (NETA et al., 2009). Disseminam-se então no interior de macrófagos ou livres através do sangue ou linfa, alojando-se em locais com abundância de células mononucleares fagocitárias, esquivando-se da resposta imune e podendo perdurar por períodos prolongados (HARMON et al., 1988; GORVEL & MORENO, 2002; XAVIER et al., 2009). A produção de enzimas antioxidantes e de guanosina 5’ monofosfato-GMP e adenina, concendem que estas bactérias sobrevivam nas células de defesa, visto que, dificultam a união entre lisossomo e fagossomo, e consequentemente, impossibilitando na fagocitose ocorra a degranulação dos macrófagos, assim como sua eliminação (ARESTÉGUI et al., 2001; NETA et al., 2009). Estas bactérias ao proliferarem, também podem ocasionar alterações como hiperplasia linfóide, esplenomegalia e hepatomegalia (MATRONE et al., 2009).

A *Brucella* spp. tem predileção pelo útero gravídico (trofoblastos), glândulas mamárias, articulações e órgãos reprodutores masculinos, os quais apresentam abundância de elementos necessários para seu metabolismo, como eritritol (álcool polihídrico de quatro carbonos) (CARTER & CHENGAPPA, 1991; XAVIER et al., 2009). A elevação dos níveis dessa substância próxima ao parto aumenta a capacidade da infecção e multiplicação do agente (LAGE et al., 2008b). Em decorrência do processo inflamatório vários tecidos são lesionados levando ao descolamento dos cotilédones placentários e prejuízos a circulação materno-fetal o que acarreta em desde nascimento de bezerros subdesenvolvidos ao aborto (XAVIER et al., 2009).

A *Brucella* é transmitida principalmente via infecção oral, além de vias aéreas respiratórias, trato genital, pele e conjuntivas (ACHA & SZYFRES, 2001). Através do parto ou aborto, os animais infectados disseminam as bactérias contaminando outros animais e o ambiente. As fêmeas positivas tornam-se cronicamente portadoras após o primeiro aborto e assim, eliminam a bactéria pelo leite, urina e descargas uterinas no decorrer dos próximos partos, com ou sem aborto. Os abortos deixam de ocorrer depois da terceira gestação pós-infecção, o que se deve ao desenvolvimento de imunidade celular e diminuição da necrose dos placentomas (PAULIN & FERREIRA NETO, 2003). Animais infectados eliminam altas quantidades de *Brucella* no aborto ou parto, o que em conjunto com a resistência ambiental elevada do agente configuram-se como método essencial de infecção para os suscetíveis, além do fator de lamber ou cheiras crias ou fetos favorecer a transmissão (SILVA et al., 2005). Embora encontrada no sêmem, a transmissão via monta natural destas bactérias pelos machos é infrequente, visto que, as barreiras naturais da vagina dificultam a infecção, o que não ocorre na inseminação artificial onde deposita-se o sêmen diretamente no útero, favorecendo a sobrevivência e multiplicação (BRASIL, 2006; LAGE et al., 2008b). A água e alimentos contaminados também podem transmitir o agente (ACHA & SZYFRES, 2001).

Situações com sombra, umidade, baixas temperaturas e pH neutro ampliam a sobrevicência das *Brucellas* spp. (PAULIN & FERREIRA NETO, 2003; BRASIL, 2006). A resistência do agente em água, restos de abortos, fezes e solo pode chegar a mais de seis meses (BRASIL, 2006; LUCERO et al., 2008), além de manter-se viável por meses em leite e derivados (OMER et al., 2000). Estas bactérias são inativadas através da pasteurização entre 10 e 15 segundos e a destruídas rapidamente pela desinfecção com hidróxido de sódio 2%, compostos de ortofenóis 3-5%, mercuriais e álcool 70% (PAULIN & FERREIRA NETO, 2003; CASTRO et al., 2005; OMER et al., 2000). A introdução de animais infectados é o principal meio de contaminação de rebanhos, tornando-se imprescindível à compra de animais

de rebanhos livres ou testados periodicamente.

O diagnóstico da brucelose por *B. abortus* pode ser realizado por métodos diretos (imunohistoquímica e PCR) que identificam o agente ou por métodos indiretos ou sorológicos, sendo esses os mais utilizados devido detectar anticorpos contra *B. abortus* (FAVERO et al., 2008), em soro sanguíneo, leite, muco vaginal e sêmen. No Brasil, o Programa Nacional de Controle e Erradicação da Brucelose e da Tuberculose Animal (PNCEBT) estabeleceu como oficiais os testes de Antígeno Acidificado Tamponado (AAT), 2-Mercaptoetanol (2-ME), Fixação de complemento (FC), e Polarização Fluorescente (FPA). O primeiro é considerado teste de triagem e os três últimos como confirmatórios (BRASIL, 2006), sendo o (FPA) utilizado como teste único ou como teste confirmatório em animais reagentes ao teste do AAT ou inconclusivos ao teste do 2-ME (RANGEL, 2017).

O controle da brucelose visa diminuir constantemente o número de focos da doença, controlar a movimentação de animais de reprodução e aumentar o número de propriedades certificadas como livres da doença através do diagnóstico, sacrifício dos positivos para doença e adoção de medidas ambientais (PAULIN & FERREIRA NETO, 2003). A desinfecção e uso de piquetes de parição contribuem para redução ambiental do número de batérias vivas. A vacinação tem o intuito de redução da prevalência da doença a baixos custos, sendo que a cobertura vacinal é em torno de 80% por rebanho. Duas vacinas vivas atenuadas são recomendadas pela OIE, a B19 e a vacina não indutora de anticorpos aglutinantes (amostra RB51). A RB51 é utilizada pelo estado de Santa Catarina devido à baixa prevalência verificada no estado, onde são vacinadas fêmeas maiores de oito meses de idade, fêmeas adultas não reagentes a testes diagnósticos e em estabelecimentos com foco de brucelose identificados. As duas vacinas induzem boa imunidade celular (BRASIL, 2006).

Por ser uma zoonose de grande importância e uma doença que ao longo dos séculos tem trazido grandes prejuízos à pecuária, os países têm adotado medidas de controle e erradicação da doença, através da vacinação total de rebanhos, testes diagnósticos e o sacrifício sanitário de animais positivos (DEL FAVA et al., 2003). No Brasil, o PNCEBT visa reduzir os impactos econômicos e na saúde humana e animal, através da redução da prevalência e a incidência de novos focos da doença e aumento do número de propriedades livres ou monitoradas para oferta de produtos com menores riscos sanitários (BRASIL, 2006).

1.1.2 *Brucella ovis*

A brucelose ovina é causada pela bactéria *B. ovis* acomete naturalmente ovinos, raramente cervídeos (*Cervus elaphus*), assim como animais de laboratórios e caprinos em

infecção experimental (RIDLER, 2002). Apresenta distribuição mundial e não é considerada zoonótica (HOMSE, 1995). No entanto, é responsável por elevados prejuízos por comprometer a saúde reprodutiva, a qual é de vital importância para a produção e a produtividade dos rebanhos (MARTINS et al., 2012), além de, quando diagnosticada nos rebanhos constituir uma barreira potencial para o comércio de animais e produtos (WHO, 1997). A enfermidade tem sido evidenciada na maioria dos países onde a ovinocultura apresenta importância economicamente, embora controlada e erradicada por alguns países, assim como desconhecimento em alguns países (ROBLES, 1998).

A *B. ovis* é transmitida principalmente por via venérea, por meio do sêmen (ESTEIN, 1999), como demonstrado por Brown et al. (1973). Ela adere-se então à zona pelúcida do óvulo, podendo ser disseminada amplamente por meio da técnica de transferência de embriões (WOLFE et al., 1988). Além disso, palhetas de sêmen constituem outra fonte de disseminação (RIDLER, 2002), assim como a importação de reprodutores (LUNAMARTÍNEZ & MEJÍN-TERAN, 2002). O diagnóstico apenas por palpação testicular é difícil, visto que, existem outras bactérias causadoras de epididimite (BLASCO & BARBERÁN, 1990).

A *B. ovis* produz infecção subclínica ou clínica, de caráter crônico, assim como produz lesões genitais (epididimite, orquite e sêmen de qualidade variável) (MEGID et al., 2010). Como consequência, os machos podem desenvolver subfertilidade ou infertilidade (CARVALHO JUNIOR et al., 2010), e as fêmeas podem abortar ou o cordeiro vir a óbito (XAVIER et al., 2009). As lesões iniciam com a penetração do agente na mucosa, posteriormente a bactéria é conduzida livre ou no interior dos macrófagos por meio da corrente linfática até os linfonodos regionais, local de multiplicação ativa e onde permanece por dias a meses. Durante a fase aguda, ao atingir a circulação sanguínea alcança todo organismo animal, principalmente para órgãos ricos em células fagocitárias ocasionando hiperplasia linfóide, granulomas difusos, esplenomegalia, hepatomegalia e endocardite (PAULIN & FERREIRA NETO, 2003; BRASIL, 2006; LIRA & MEGID, 2009). No entanto, a maioria das lesões concentram-se nos órgãos genitais conforme já mencionado (GUL & KHAN, 2007). Na fase crônica, após o segundo mês da infecção, a bactéria encontra-se unicamente nos órgãos reprodutórios e às vezes nos rins (ROBLES, 1998). No epidídimo, a *B. ovis* produz extravasamento de espermatozóides no interstício causando pela forte reação inflamatória, e assim formação de granulomas espermáticos (ROBLES, 1998).

Apesar da *B. ovis* ter sido isolada em carneiros em diversos locais, o sêmen é mais importante forma de excreção e transmissão do agente. A infecção de fêmeas prenhes gera

uma resposta inflamatória e por consequência, placentite. Embora a bacteremia não se mantenha de uma estação reprodutiva para outra (ROBLES, 1998), os problemas são enormes. Os sinais clínicos da infeção natural e experimental são similares, com lesões nos órgãos reprodutivos relacionados a degeneração, atrofia e mineralização testicular, entre outros (PAOLICCHI et al., 2000). Silva et al. (2011) evidenciou na infecção em modelo murino uma elevada colonização bacteriana em baço e fígado, com lesões inflamatórias e esplenomegalia resultantes da infeção sistêmica, contudo, uma baixa colonização do sistema reprodutivo e as alterações histológicas restritas evidenciaram a ausência de tropismo do organismo pelo trato genital masculino de camundongos.

A prova sorológica mais empregada no diagnóstico da infecção por *B. ovis* é a Fixação de complemento (FC). Testes baseados em aglutinações não são utilizados nesse caso, visto que a *B. ovis* é rugosa e não forma suspensões estáveis (ROBLES, 1998). O MAPA recomenda a triagem por meio da Imunodifusão em Gel de Ágar (IDGA) e a confirmação dos reagentes por meio da FC (BRASIL, 2004). A identificação e abate sanitário dos positivos são medidas preconizadas para o controle, já que não existe tratamento para a enfermidade (ESTEIN, 1999).

1.2 ESTRESSE OXIDATIVO

1.2.1 Radicais livres e defesas antioxidantes

Define-se por radicais livres qualquer átomo, molécula ou fragmentos moleculares contendo um ou mais elétrons desalinhados nas suas camadas de valência (FERREIRA & MATSUBARA, 1997), gerando moléculasinstáveis, com meia vida reduzidas e altamente reativas (BARREIROS et al., 2006). Os radicais derivados do metabolismo do oxigênio e nitrogênio (radical hidroxila (OH•), ânion superóxido (O2-) e óxido nítrico (NO•)) representam a classe mais importante de espécies radicais geradas nos sistemas biológicos (BARREIROS et al., 2006). Existem compostos tão reativos quanto os radicais livres e que por não apresentam elétrons desalinhados em sua última camada, não podem ser classificados da mesma forma (FERREIRA & MATSUBARA, 1997), sendo então nomeadas como espécies reativas de oxigênio (EROs) ou espécies reativas do nitrogênio (ERNs) (Peróxido de hidrogênio (H2O2), oxigênio singlet (1O2) e o peroxinitrito (ONOO-)) (HALLIWELL, 2007).

Durante o processo de metabolismo celular, estas espécies reativas são constantemente produzidas pelo organismo, e desempenham, quando em baixas ou moderadas concentrações,

funções fisiológicas significativas que incluem a regulação do tônus vasculares e fagocitose por exemplo (DRÖGE, 2002). Entretanto, em concentrações excessivas são prejudicais, causando graves danos às macromoléculas biológicas, como lipídeos, proteínas e DNA (BARREIROS et al., 2006). Desta forma, o mecanismo de defesa antioxidante (enzimáticos e não enzimáticos) são estimulados com o intuito de delimitar os níveis dessas espécies reativas e proteger as células contra efeitos da oxidação (FERREIRA & MATSUBARA, 1997). Fisiologicamente, a formação de espécies reativas e a sua eliminação ou neutralização pelo sistema antioxidante é equilibrada. No entanto, o desequilíbrio entre os níveis de antioxidantes e pró-oxidantes, com o preponderância dos oxidantes acarretam em uma situação denominada de estresse oxidativo (BARBOSA et al., 2010).

O estresse oxidativo pode ocorrer em resposta ao aumento da geração de espécies reativas ou depleção dos antioxidantes de defesa. Em consequência disto, macromoléculas e diversas estruturas celulares são danificadas, alterando o funcionamento de tecidos e órgãos caso não haja o reparo (BARREIROS et al., 2006). Doenças com desordens neurodegenerativas como Parkinson e Alzheimer tem sua patogenia associadas com o estresse oxidativo (EMERIT et al., 2004). A patogênese da brucelose também tem sido relacionada ao aumento do estresse oxidativo e depleção de antioxidantes (SEREFHANOGLU et al., 2009). Melek et al. (2006) ao investigarem o efeito do estresse oxidativo e da inflamação na infecção por *B. melitensis* a longo prazo observação uma indução da peroxidação lipídica e a produção hepática e esplênica de óxido nítrico nos primeiros dias da infecção, com diminuição subsequentemente. A peroxidação lipídica, oxidação protéica e o dano no DNA celular são alguns dos danos oxidativos ocasionados pelas EROs (CHIHUAILAF et al., 2002; HALLIWELL e GUTTERIDGE, 2007). No entanto, o combate aos agentes infecciosos através da produção de EROs é muito relevante, pois auxilia o sistema imune contra a invasão do microorganismo. De acordo com a literatura, as alterações oxidativas são danosas às estruturas celulares dos microorganismos, o que auxilia o sistema imune na defesa contra a invasão (MARR & MULLER, 1995).

1.2.2 Peroxidação lipídica

O processo em que as EROs lesionam os ácidos graxos polinsaturados dos fosfolipídeos das membranas das células é conhecido por peroxidação lipídica, e resulta na desintegração das membranas, e assim possibilitando a entrada dessas espécies nas estruturas intracelulares. A liberação os ácidos graxos não saturados ocorre através da ação da fosfolipase que desintegra do fosfolipídeos ao ser ativada pelas espécies tóxicas

(HALLIWELL & GUTTERIDGE, 1989). Além disso, a lipoperoxidação tem sido inversamente relacionada ao crescimento tumoral, por apresentar papel importante na proliferação celular (GONZALEZ, 1992). O início da peroxidação lipídica se dá quando as EROs atacam ligações duplas ou triplas de ácidos graxos poli-insaturados, alterando sua conformação química e consequentemente a integridade estrutural ocasionando perda da fluidez e aumento da permeabilidade a íons das células (CHIHUAILAF et al., 2002). Além disso, esta reação pode formar Fe^{3+}, que pode reagir com peróxidos lipídicos forma os radicais peroxilas e Fe^{2+}, consituindo um problema, visto que, a peroxidação lipídica da membrana ligada à liberação do Fe^{2+} leva a hemólise de eritrócitos (HALLIWELL & GUTTERIDGE, 2007; KOURY & DONANGELO, 2003), o que poderia explicar os casos de anemia relacionado à brucelose. São formados também durante lipoperoxidação gases de hidrocarbonetos e aldeídos, como o malondialdeído (MDA) (HALLIWELL & GUTTERIDGE, 2007). Como o metabolismo lipídico pode ter envolvimento também na invasão das células hospedeiras, a mensuração dos níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS) é o método mais utilizado para avaliação da peroxidação lipídica tecidual (ESTERBAUER, 1993), onde danos teciduais decorrentes de sua elevação e relacionados a diversas condições patológicas (HALLIWELL & CHIRICO, 1993). Apesar disso, a mensuração de níveis de TBARS é consideradare uma das técnicas questionadas por pesquisadores internacionais, que argumentam que TBARS hidrolisa outras coisas além de MDA.

1.2.3 Mecanismos antioxidantes

Antioxidantes são classificados como qualquer substância que, mesmo em pequenas concentrações em relação aos substratos oxidáveis, retardam ou inibem as reações de oxidação e podem agir em diferentes níveis da sequência oxidativa. O sistema de proteção antioxidante tem como função equilibrar os agentes pró-oxidantes e antioxidantes através da coibição dos efeitos deletérios das EROs (CHIHUAILAF et al., 2002; HALLIWELL & GUTTERIDGE, 2007). A primeira linha de defesa endógena de neutralização das EROs é realizada pelo sistema antioxidante enzimático, constituído pela superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx), as quais procuram manter baixas concentrações de radical superóxido e de peróxidos de hidrogênio nas células, o que evita a formação da radical hidroxila (HALLIWELL & GUTTERIDGE, 2007).

De acordo com a literatura, a SOD é uma metaloenzima, que atua removendo o radical superóxido. Tem como co-fatores zinco, cobre e manganês, e é responsável por transformar

dois ânions de radical superóxido (O_2 •) em um peróxido de hidrogênio menos reativo que o anterior, catalisando a dismutação a peróxido de hidrogênio. Já a CAT ou da GPx degram então o peróxido de hidrogênio, gerando H_2O e O_2, que podem atravessar a membrana nuclear em forma de H_2O_2 e ocasionar através de reações enzimáticas danos na molécula de DNA (ANDERSON, 1996). A enzima CAT está presente na maioria das células aeróbicas, e é encontrada em principalmente no fígado, rins e eritrócitos, assim como em menor quantidade no cérebro, coração e músculo esquelético (HALLIWELL & GUTTERIDGE, 1989). São co-fatores desta enzima o ferro e a vitamina E, além disso, a CAT tem como função evitar o acúmulo de metahemoglobina e decompor o peróxido de hidrogênio em água e oxigênio molecular (GAETANI et al., 1989).

1.3 SISTEMA PURINÉRGICO

A sinalização purinérgica é alvo de estudos envolvendo vias de sinalização em diversos tecidos, desencadeando variados efeitos celulares e também, devido seu papel de modular vários processos biológicos. Esse sistema está envolvido em mecanismos neuronais e não neuronais e em eventos de curta e longa duração, como a secreção exócrina e endócrina, resposta imune e inflamatória, dor, agregação plaquetária, vasodilatação mediada pelo endotélio, proliferação e morte celular (BURNSTOCK, 2007; ROBSON et al., 2006; SCHETINGER et al., 2007). Os componentes principais deste sistema são os nucleotídeos e nucleosídeos extracelulares, seus receptores através dos quais estes nucleotídeos e nucleosídeos exercem seus efeitos e as ectoenzimas responsáveis pela regulação dos níveis extracelulares dessas moléculas (YEGUTKIN, 2008).

1.3.1 Nucleosídeos e nucleotídeos de adenina

Os nucleosídeos (iosina e adenosina) são moléculas que resultam da união de uma base púrica ou pirimídica com uma pentose, que, ao serem fosforilados por quinases específicas tornam-se nucleotídeos (ATKINSON et al., 2006). Os nucleotídeos de adenina (ATP, ADP e AMP) são considerados importantes moléculas de sinalização em diversos tecidos (YEGUTKIN, 2008), envolvidas em várias funções fisiológicas detalhadas a seguir (RALEVIC & BURNSTOCK, 1998). O ATP, ADP e a adenosina regulam processos associados à tromboregulação, modulação de respostas imune e sinalização de vias fundamentais para o funcionamento e desenvolvimento do sistema nervoso (BURNSTOCK, 2002), além de influenciarem respostas vasomotras e controle a atividade plaquetária

(ATKINSON et al., 2006), por exemplo. O ADP é o principal agonista relacionado ao recrutamento e agregação plaquetária (REMIJN et al., 2002) ao passo que, o ATP quando em concentrações elevadas é inibidor competitivo das ações mediadas pelo ADP (SOSLAU & YOUNGPRAPAKORN, 1997). A adenosina por sua vez, modula os tônus vascular, além de inibir a agregação plaquetária (ANFOSSI et al., 2002). A ativação ou inibição da resposta imune e inflamatórias também possuem envolvimento da adenosina e do ATP (BOURS et al., 2006), que de acordo com literaturas dos últimos 10 anos tem sido associado a patogenia de doenças infeciosoas.

O ATP é uma molécula sinalizadora do sistema purinérgico, presente em todas as células e com envolvimento na regulação de vários processos fisiopatológicos extracelulares. Após despolarização neuronal, o ATP acondicionado nas vesículas de terminações sinápticas é liberado e atua nos purinoreceptores na membrana pós-sináptica (RALEVIC & BURNSTOCK, 1998). Fisiologicamente ou em resposta a danos celulares (hipóxia e injúrias) o ATP pode ser liberado nos terminais pré e pós-sinápticos (BURNSTOCK, 2008), assim como durante a resposta imune ou por células do endotélio danificadas, visto que, linfócitos T e eritrócitos secretam ATP via canais de panexina-1. Através destes canais, a liberação de ATP é controlada durante os mecanismos de apoptose e permeabilidade da membrana (LOCOVEI et al., 2006), contribuindo para o desencadeamento da resposta inflamatória (MARIATHASAN & MONACK, 2007). De acordo com a literatura, o ATP é um neurotransmissor excitatório nas sinapses nervosas purinérgicas, podendo ser também co-liberado com outros neurotransmissores como a acetilcolina e a noradrenalina (BURNSTOCK, 2006). O ATP atua através de purinoreceptores do tipo P2 (P2X e P2Y), tanto como transmissor quanto como co-transmissor (BURNSTOCK, 2012), e conforme sua concentração extracelular e tipo de receptor envolvido, ele pode ter ações pró e anti-inflamatórias (DI VIRGILIO et al., 2009).

Situações pró-inflamatórias geram estimulação e proliferação de linfócitos, que são essenciais para secreção de citocinas dependentes das células T, entre elas as relacionadas à indução de resposta imune a antígenos estranhos como o INF-γ e IL-2. Por exemplo, na contração do músculo liso, neurotransmissão, inflamação e dor (SNEDDON et al., 1999), além da recrutação de monócitos para tecidos alvo (VENTURA & THOMOPOULOS, 1995), produção de IL-1β e TNF-α por macrófagos (ELSSNER et al., 2004) e migração e diferenciação de células dendríticas (LA SALA et al., 2003) também tem participação. Todavia, quando em baixas concentrações extracelulares, o ATP apresenta papel imunossupressor, inibindo a proliferação de células T e, coibindo a liberação de citocinas pró-

inflamatórias (GESSI et al., 2007). Nessas condições, ocorre um aumento da sua afinidade por receptores do tipo P2Y, localizados na superfície dos linfócitos, e que quando estimulados promovem uma desregulação na expressão e liberação de citocinas pró-inflamatórias, estimulando uma resposta Th2 e liberando citocinas anti-inflamatórias, processo que promove um efeito protetor contra danos teciduais excessivos (BOURS et al., 2006). Estes danos ocorrem em situações com altas concentrações de ATP extracelular, atuando então como molécula citotóxica, formando grandes poros na membrana plasmática que levam a morte celular (FILIPPINI et al., 1990). Em situações patológicas, a liberação de ATP e a expressão de receptores purinérgicos pelas células é elevada (GUIDO et al., 2008), sendo que a regulação da sua concentração extracelular é realizada por ectonucleotidases que catalisam e fazem sua conversão ADP na cascata purinérgica tradicional (ROBSON et al., 2006). O ADP é conhecido principalmente pela importante participação na regulação da hemostasia, o que não foi foco de nosso estudo.

A adenosina é considerada um neuromodulador, envolvido na síntese de ácidos nucléicos, metabolismo de aminoácidos e modulação do estado metabólico da célula (CHEN, 2009). Esta molécula também é responsável por regular a liberação de neurotransmissores (DUNWIDDIE & MASINO, 2001), o metabolismo celular e efeitos fisiológicos, como a apoptose, necrose e proliferação celular. Molécula importante na mediação de ações anti-inflamatórias e imunossupressoras pela inibição da produção de citocinas pró-inflamatórias e da proliferação de linfócitos (GESSI et al., 2007). Além disso, a adenosina pode sinalizar dano celular, porém apresenta ações antagônicas as do ATP (BOURS et al., 2006). Em condições patológicas, a adenosina desempenha papel protetor, defendendo o organismo ao modular a liberação de neurotransmissores e atuar em casos de lesão tecidual excessiva frente à inflamação, como regulador endógeno da imunidade inata (DESROSIERS et al., 2007). Embora presente extracelularmente em baixas concentrações, em condições de estresse metabólico os níveis extracelulares de adenosina elevam-se (BARALDI & BOREA, 2000), conforme já registrado em doenças infeciosas. As ações da adenosina fisiologicamente decorrem da ocupação dos receptores de superfície e ativação de vias de sinalização intracelular (HASKÓ & CRONSTEIN, 2004), onde níveis nanomolares ativam receptores A1 e A2A. De acordo com a literatura, ocorrem elevações extracelulares na faixa de micromolar durante períodos de inflamação, hipóxia ou isquemia ativam subtipos A2B e A3 (OLAH & STILES, 1995; BARALDI & BOREA, 2000).

Em resumo, as funções dos nucleotídeos e nucleosídeos de adenina são reguladas por receptores purinérgicos em diferentes tipos de células (YEGUTKIN, 2008), sendo para

nucleotídeos existem dois grupos de receptores P2X e P2Y, onde o P2X é um receptor acoplado a canais iônicos e P2Y acoplado à proteína G. Já os receptores da adenosina incluem quatro tipos, isto é, A_1, A_{2a}, A_{2b}, A_3, os quais são proteínas transmembrana ligadas à proteína G (HASKÓ et al., 2008; YEGUTKIN, 2008). Conforme já mencionado, a regulação desses é realizada pelas enzimas purinérgicas, detalhadas a seguir.

1.3.2 Ectonucleotidases e adenosina desaminase

A sinalização purinérgica é controlada por enzimas ancoradas na membrana celular ou no meio intersticial, dentre estas enzimas, destacam-se a ecto-nucleosídeo trifosfato difosfohidrolase (E-NTPDase), ecto-nucleotídeo pirofosfatase (E-NPPs), 5'-nucleotidase e adenosina desaminase (ADA) (ROBSON et al., 2006; YEGUTKIN, 2008). Estas enzimas são capazes de controlar a disponibilidade de moléculas como ATP e ADO aos seus receptores específicos (ZIMMERMANN, 2011), os quais estão envolvidos em diversas funções descritas na seção anterior.

As ectonucleotidases formam uma cadeia enzimática que inicia com a ação da E-NTPDase e da E-NPP que hidrolisam o ATP e o ADP, formando AMP (ZIMMERMANN et al., 2008). O AMP em seguida é hidrolizado pela enzima 5'-nucleotidase hidrolisa formando adenosina, a qual por fim é degradada pela ação da ADA em inosina (YEGUTKIN, 2008). A disponibilidade de ligantes como o ATP, ADP, AMP e adenosina é moderada pela ação das ectonucleotidases tanto para receptores de nucleotídeos e nucleosídeos como sua extensão e a duração da ativação do receptor (CHEN E GUIDOTTI, 2001). Esta via enzimática apresenta dupla função pois remove a molécula sinalizadora (ATP) e gera outra molécula de regulação (adenosina) (ABBRACCHIO et al.,2009).

A enzima ADA atua conjunto com outras enzimas na degradação dos nucleotídeos e nucleosídeos de adenina (YEGUTKIN, 2008). Por regular as concentrações de adenina a esta enzima atua na deaminação da adenosina em inosina, seu metabólito inativo (FRANCO et al., 1997). Altos níveis desta enzima são encontrados em órgãos sistema linfóide como linfonodos, baço e timo, e em menores quantidades nos eritrócitos, além de ter sido detectada em várias superfícies celulares, expressando atividade em tecidos periféricos e no SNC, com variabilidade em áreas cerebrais conforme as vias purinérgicas (GEIGER et al., 1986; FRANCO et al., 1986; 1997). A ADA exerce papel importante na diferenciação e proliferação de linfócitos T (CODERO et al., 2001), além disso, pode atuar na maturação de células vermelhas na superfície das células hematopoiéticas (ARAN et al., 1991). De acordo com pesquisadores, a deficiência de ADA pode contribuir para condições patológicas

(ALDRICH et al., 2000), sendo assim, sua atividade vem sendo utilizada na monitoração de diversas patologias envolvendo o sistema imune e alterações em sua atividade indicam distúrbios imunológicos (POURSHARIFI et al., 2009). O ancoramento da ADA na membrana celular é realizado por duas proteínas, a CD26 de membrana, e o receptor de adenosina A1 (YEGUTKIN, 2008), e essa interação pode ser importante no contato entre diferentes tipos celulares e expressiva para o desenvolvimento do sistema nervoso e imune (FRANCO et al., 1997). A ADA divide-se em duas isoformas, isto é, ADA1 e ADA2, sendo que ADA1 é predominante em tecidos, enquanto que, a ADA2 encontra-se principalmente em soro e estimula células-T (BURNSTOCK, 2006). A ADA1 é uma proteína de localização principalmente citosólica e encontrada em todo o organismo, inclusive na superfície de macrófagos, linfócitos B e em alguns linfócitos T e, pode combinar-se com a proteína combinante (CD26). Esta junção constitui uma ecto-ADA incumbida de controlar os níveis de adenosina extracelulares (FRANCO et al., 1997). A ADA1 também pode atuar como uma ecto-enzima ancorada aos receptores de adenosina (A1 e A2b), intercedendo em processos de sinalização deste nucleosídeo neuromodulador (ROMANOWSKLA et al., 2007). A ADA2 plasmática não apresenta funções claras, sugerindo-se que a mesma pode ser secretada por monócitos ativados em processos inflamatórios (IWAKI-EGAWA et al., 2004). A elevação da atividade de ADA no soro tem sido observada em várias patologias como tuberculose (KAISEMANN et al., 2004) e leishmaniose (VIJAYAMAHANTESH et al., 2016), desta forma, esta enzima tem sido utilizada como marcador de infecção e para o acompanhamento do curso das mesmas.

1.4 SISTEMA COLINÉRGICO

O sistema colinérgico é uma das principais vias de modulação do sistema nervoso central (SNC) e está envolvido funções vitais como aprendizado, memória, organização do movimento e controle do fluxo sanguíneo cerebral (MESULAM et al., 2002), além de funções cognitivas, como atenção e memória (GOLD, 2003). No sistema nervoso autônomo (SNA) é responsável por controlar a contração da musculatura lisa gástrica (ROGERS et al., 1999) e a frequência cardíaca (MENDELOWITZ, 1999). Este sistema é alvo de inúmeras pesquisas, principalmente relacionadas às vias de sinalização intercelular e intracelular, através da ativação dos receptores colinérgicos. Neste contexto, destaca-se como principal constituinte deste sistema a acetilcolina (ACh), molélula regulada por enzimas conhecidas como colinesterases: a acetilcolinesterase (AChE) e butirilcolinesterase (BChE),

responsáveis pela regulação de diferentes situações fisiológicas, estando entre elas à modulação de processos inflamatórios (DAS, 2007).

A ACh é considerada um neurotransmissor de sinapses e junções neuroefetoras colinérgicas dos SNC e sistema nervoso periférico (SNP). De acordo com a literatura, sua síntese ocorre no citosol dos neurônios a partir da acetil coenzima-A (acetil-CoA) de origem mitocondrial e da colina proveniente da fenda sináptica extracelular. Está combinação é catalisada pela ação da enzima colina acetiltransferase (ChAT) (PRADO, 2002), e após ser sintetizada a ACh é carreada pelo transportador vesicular de acetilcolina (VAChT) até as vesículas sinápticas, onde fica armazenada até a sua liberação (RAND, 2007). Quando ocorre o impulso nervoso, a membrana pré-sináptica é despolarizada, aumentando a condutância de cálcio, fator que favorece sua entrada no axônio. Portanto, a liberação da ACh varia conforme a concentração de cálcio (PRADO et al., 2002). Na sequencia, as vesículas rompem e o contéudo (ACh) extravasa na fenda sináptica, pondendo difundir-se no espaço extracelular, combinar-se com receptores colinérgicos pós e pré-sinápticos, ou ainda, ser degradada em colina e ácido acético pelas colinesterases. Os receptores colinérgicos são classificados como nicotínicos e muscarínicos (nAChR e mAChR), atuam a nível neurônio pré-sináptico transmitindo sinais (RANG et al., 2004). Ambos receptores são formados por diversas subunidades α e β (α2 - α10, β2 - β4), contudo, os nAChR estão mais disseminados facilitando que outros neurotransmissores sejam liberados (RANG et al., 2004). Importante ressaltar que metade da colina produzida é então recaptada pelo axônio terminal e reaproveitada na biossíntese de ACh (FERREIRA et al., 2008; ABREU-VILLACA et al., 2011).

A AChE e BuChE são enzimas presentes em tecidos colinérgicos e não colinérgicos, assim como sangue e outros fluídos corporais. Segundo pesquisadores, estas enzimas são classificadas de acordo com suas propriedades catalíticas, especificidade aos substratos, sensibilidade a inibidores e distribuição tecidual (CHATONNET & LOCKRIDGE, 1989). A AChE é uma importante enzima responsável por controlar a transmissão do impulso nervoso através da sinapse colinérgica. Essa enzima realiza hidrólise rápida e inativando a ACh, o que faz com que a concentração deste neurotransmissor nas sinapses seja modulada (SOREQ & SEIDMAN, 2001), pois é amplamente distribuída no SNC com destaque para os neurônios colinérgicos, próximo às sinapses colinérgicas, e em altos níveis na junção neuromuscular (MASSOULIÉ et al., 1993). A enzima está presente em eritrócitos, linfócitos e plaquetas de mamíferos (SILVA, 1998), envolvida na regulação das funções imune

(KAWASHIMA & FUJOO, 2000) e eritropoiese. Importante ressaltar que sua inibição ou ativação pode afetar drasticamente o cérebro e outros órgãos (MESULAN et al., 2002).

A BChE é uma enzima multifuncional produzida pelo fígado (TAYLOR & BROWN, 1999), conhecida a décadas pela sua capacidade de catalisar a hidrólise de ésteres de colina endógena (KALOW & STARON, 1957) como a acetilcolina, propionilcolina e especialmente a butirilcolina (SILVER, 1974). Essa enzima também atua substituindo a AChE em efeitos compensatórios, quando ausente ou comprometida, para manter e regular a transmissão colinérgica (LI et al., 2000). Apesar de ser encontrada em diversos sistemas, suas funções biológicas deixam dúvidas, embora capaz de hidrolisar a ACh, a BChE não possui substrato natural específico (MASSOULIÉ et al., 1993), sugerindo seu envolvimento na detoxificação de compostos naturais. Ambas as enzimas atuam como marcadores inflamatórios (DAS, 2007), apresentando atividade elevada no plasma e tecidos de pacientes com doença de Alzheimer e Diabetes mellitus por exemplo (GIACOBINI, 2003; SRIDHAR et al., 2005), o que consequente levaria a redução dos níveis de ACh. A ACh por sua vez, inibe a produção de fator de necrose tumoral (TNF), interleucina-1 (IL-1) e fator inibidor da migração de macrófagos (MIF), além de modular a resposta imune e a neurotransmissão por regular os níveis da serotonina, dopamina e de outros neuropeptídeos (RAMIREZ et al., 1997). De acordo com pesquisadores, a ACh está envolvida na via colinérgica anti-inflamatória, visto que, é capaz de inibir que macrófagos secretem TNFα, IL-1β, assim como uma série de outros mediadores inflamatórios (TRACEY, 2002; 2009).

1.5 METABOLISMO ENERGÉTICO

As enzimas do metabolismo energético conhecidas como creatina quinase (CK), piruvato quinase (PK) e adenilato quinase (AK) são responsáveis por regular as concentrações de ATP e envolvidas na homeostase e manutenção tecidual (DE FRANCESCHI et al., 2013). O papel da CK é significativo em células com necessidades energéticas elevadas, como coração, cérebro e músculo esquelético, mantendo a homeostase nestes tecidos (SEGAL et al., 2007). Alterações nesta enzima estão relacionadas a inúmeras patologias, sendo considerada um importante marcador inflamatório (TOREN et al., 1994). A AK é responsável pela interconversão de ATP em ADP e AMP (DZEJA e TERZIC, 1998), por catalisar a reação de transferência de alta energia entre estes nucleotídeos. A PK cataliza a transferência de um grupo fosforil do fofoenolpiruvato para o ADP formar piruvato e ATP, além disso, é considerada uma das enzimas regulatórias da via glicolítica,

chave para o metabolismo celular (VALENTIN et al., 2000) e regulação do sistema imune do hospedeiro (SAUVAGE et al., 2009). Deficiências nestas enzimas estão relacionadas a desordens celulares e de regiões com maiores demandas de ATP (RECH et al., 2008).

Diversos processos biológicos como neutransmissão, contração muscular, função cardíaca, agregação plaquetária, metabolismo do glicogênio hepático tem participação do ATP extracelular. Além disso, em resposta a danos ou estímulos celulares por ação de patógenos há ocorrência de liberação de ATP extracelular, estabelecendo-se um processo inflamatório (LANGSTON et al, 2003). A manutenção da homeostase energética é essencial para a sobrevivência celular normal (FOO et al., 2012) e a oxidação das moléculas energéticas é então regulada para manutenção da homeostática do ATP celular, evitando distúrbios como hipotireoidismo, obesidade e infarto do miocárdio (MARKS et al., 2007). Para tanto, reações enzimáticas de trocas de grupos fosforil de alta energia em associação com metabolismo energético mitocondrial se fazem necessárias para que o ATP seja produzido e consumido adequadamente pelas células (GLORIA-BOTTINI et al., 2011). Estudo realizado com camundongos infectados com *T. gondii* comprovou alterações nos níveis de ATP no encéfalo conforme a fase da doença, elevando-se na fase aguda e reduzindo na fase crônica da infecção (TONIN et al., 2014a).

Neste contexto, sabendo-se da importância da brucelose na saúde pública e na produção animal, estudos que busquem conhecer a influência da infecção por *B. abortus* e *B. ovis* na atividade das enzimas do sistema purinérgico, colinérgico, metabolismo energético e nos parâmetros de estresse oxidativo são de extrema valia, visto que, possuem o intuito de contribuir no esclarecimento da patogênese da brucelose, em virtude de desempenham papéis importantes na regulação do sistema imune. Sendo assim, buscou-se estabelecer a participação destes sistemas com a brucelose natural e experimental e suas consequências para a saúde animal. Os resultados obtidos serão expostos e discutidos no decorrer desta dissertação na forma de artigos e manuscritos.

1.6 OBJETIVO GERAL

Verificar a participação ou papel do estresse oxidativo, sistema purinérgico, sistema colinérgico e metabolismo energético na patogenia da doença em vacas soropositivas para *Brucella abortus* (infeção natural) e em camundongos infectados experimentalmente com *Brucella ovis,* assim como avaliar a prevalência de *B. abortus* em vacas no oeste de Santa Catarina.

2 - CAPÍTULO II

ARTIGO E MANUSCRITO

Os resultados desta dissertação são apresentados na forma de três artigos e três manuscritos, com sua formatação de acordo com as orientações da revista ao qual foi submetido.

2.1 – ARTIGO I

Occurrence of oxidative stress in dairy cows positive for *Brucella abortus*

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Occurrence of oxidative stress in dairy cows seropositives for *Brucella abortus*

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Abstract

Bovine brucellosis is an important zoonotic disease caused by the bacterium *Brucella abortus* that leads to economic losses due to animal discard and commercial restrictions. Since positive animals for brucellosis are culled, little is known about the pathogenesis of this disease. Therefore, the aims of this study were to evaluate possible changes in the activity of deaminase adenosine (ADA) and the oxidative stress in cows seropositives for brucellosis (Experiment I), and to evaluate the seroprevalence of *B. abortus* in dairy cows from the Western state of Santa Catarina, Southern Brazil (Experiment II). The Experiment I evaluated 20 pregnant cows: ten seropositives for *B. abortus* and ten seronegatives that were used as controls. The ADA activity and markers of oxidative stress (TBARS, catalase (CAT) and superoxide dismutase (SOD)) were evaluated in these animals. A reduction in the activity of ADA and catalase enzymes in seropositive animals was observed ($p<0.001$). Conversely, there was an increase in TBARS levels and superoxide dismutase activity in cows infected by *B. abortus* ($p<0.001$). The presence of oxidative stress and a reduction of ADA might be related to the modulation of the inflammatory response. The experiment II was performed due to a high number of herds with restrictions imposed by cases of brucellosis in the state of Santa Catarina in the last two years, and thus, the seroprevalence for *B. abortus* was evaluated in 1,242 serum samples of cows of 69 herds. The serodiagnosis was performed using two tests: buffered acidified antigen and 2-mercaptoethanol. However, none of the serum samples were positive for *B. abortus*. Although we did not find seropositive animals for brucellosis in our study, the disease still requires continued surveillance, due to its economic impact, and to the oxidative stress caused by it, which may have contributed to cases of abortion in three seropositive cows (Experiment I) in the final third of the gestation.

Key words: brucellosis; cattle; inflammation; cellular damage.

1 Introduction

Bovine brucellosis is a cosmopolitan contagious disease that affects mammals, and *Brucella abortus* is the primary etiologic agent of it [1]. This disease is a zoonosis of importance to public health, as well as responsible for economic losses in animal production due to the occurrence of abortions, interference with animal fertility and birth of newborns [2]. Furthermore, herds with infected animals have restriction to market their products, as well as in animal husbandry [3]. The main consequence of bovine brucellosis is abortion, due to a series of biochemical events that, if exacerbated, may lead to fetal expulsion or embryonic death. Among some physiological mechanisms, the formation of free radicals occurs as mediators of biochemical reactions, but under pathological conditions there is an overproduction of these radicals, causing cell and tissue damage, and therefore requiring activation of the antioxidant system [4]. To minimize the effects of oxidative stress caused by oxidative/antioxidant imbalance, a series of other events occur, where purinergic enzymes have shown an important modulating role.

In response to inflammation, the levels of free radicals and other reactive oxygen species in the cellular environment may increase, and in this context, purinergic enzymes play important functions modulating the inflammatory process and avoiding greater cellular damages. Purinergic enzymes are enzymes that act to metabolize ATP, a functionally important molecule, since ATP and its metabolites act as physiological ligands in different purinergic receptors [5]. The nucleotides (ATP and ADP) are hydrolyzed by the purinergic enzymes into nucleosides, a process that leads to the hydrolysis of both in AMP, ending the ectonucleotidase cascade with the hydrolysis of the monophospated nucleotides, which results in adenosine [5], an endogenous activator of the enzymatic antioxidant system during cellular injury [6]. The enzyme adenosine deaminase (ADA) is involved in several biological functions of the organism, but it is important to emphasize that it plays an essential anti-

inflammatory role in several diseases caused by infectious agents [7], reasoning this study. The ADA actively participates in the metabolism of the adenine nucleotides, an important component of the purinergic system, acting as a central nervous system modulator, regulating cell metabolism, and participating in a range of physiological effects, such as apoptosis, necrosis, and cell proliferation [8, 9]. This nucleoside is also considered a signaling molecule of cellular damage, but with antagonistic actions to those of ATP, because it mediates anti-inflammatory and immunosuppressive effects, such as inhibition of the production of pro-inflammatory cytokines and proliferation of lymphocytes [8, 9]. Adenosine is called a "retaliatory metabolite" because it is a regulatory autocoid that is generated as a result of cellular injury or stress, interacting with specific G protein-coupled receptors on inflammatory, and immune cells to regulate their functions; and the effects of adenosine, acting on its receptors, on the functions of the cells that mediate innate immune responses [8, 9]. The physiopathological mechanism has not been definitely established but the CD4+ lymphocytes and macrophages are pointed as being accountable for higher enzymatic activity, and for this reason, ADA could be a marker of the cellular immune response [8, 9].

In several countries from Middle East and Mediterranean region, as well as Sub-Saharan Africa, China, India, Peru and Mexico, there are currently a growing number of cases of brucellosis, and Southeastern and Central Asia have the highest number of cases caused by *B. abortus* [10]. However, some Western and Northern European countries are considered free of bovine brucellosis, as well as Czech Republic, Canada, Japan, Australia and New Zealand [11, 12]. The European Union has invested in programs aimed to eradicate brucellosis. In Brazil since 2001, there are many actions being developed by the National Program for the Control and Eradication of Brucellosis and Tuberculosis (PNCEBT) [1]. Few studies are well planned and comprehensive on the situation of bovine brucellosis, even though the disease is present throughout the Brazilian territory. According to the literature, the

Brazilian state of Santa Catarina (SC) has the lowest national prevalence (0.32% of herds and 0.06% of positive animals) [13]. However, in November 2016, animals from 150 herds were placed under surveillance by the *Companhia de Desenvolvimento Agrário de Santa Catarina* (CIDASC) for being positives for brucellosis. These findings were published in the local media causing concerns to farmers and general population.

This study was designed in order to verify the sanitary status of cattle for brucellosis, as well as the consequences of the disease. Therefore, the aims of this study was to evaluate whether there is a change in the ADA activity and occurrence of oxidative stress in cows seropositives for brucellosis, as well as to evaluate the seroprevalence of *B. abortus* in dairy cows in the Western region of Santa Catarina State, Southern Brazil.

2 Material and methods

The methodology section was divided into two distinct experiments. Based on the results obtained from the Experiment I, in addition to the great repercussion of new cases of bovine brucellosis in the Western Santa Catarina state, the Experiment II was also designed.

2.1 Experiment I

2.1.1 Animals

In a herd of the Santa Catarina state, Southern Brazil, cows with history of abortion in the last third of their pregnancies and constant estrus repetitions (more than 15 cows) were serologically tested to identify antibodies against *Brucella abortus* carried out by the *Companhia de Desenvolvimento Agrário de Santa Catarina* (CIDASC), a **state governmental agency**. Firstly, the buffered plate antigen test (BPA) was used and positive samples were confirmed by the test of 2-mercaptoethanol (2-ME) [1]. With the results of serological tests for *B. abortus*, frozen sera and blood samples (-20 °C) were separated into

two groups: seropositives (n = 10) and seronegatives (n = 10). For this study, only the cows with 3 to 5 years of age, Holstein breed, on their final third of gestation (7 and 8 months) were selected. After collecting material for biochemical analyzes, three seropositives cows had abortions. The other cows had full-term calving of apparently healthy nerwborns. The farm used semi-extensive production system, i.e. cultivated pasture, concentrate, and corn silage. Access to pasture was free, prevailing perennial (*Cynodum* spp) and annual crops (*Avena strigosa* and *Lolium multiflorum*).

2.1.2 Protein determination in serum

Protein was measured by the Coomassie blue method using bovine serum albumin as the standard as described by Bradford [14]. This analysis was necessary to evaluate the markers of oxidative stress.

2.1.3 ADA analysis

Seric ADA activity was measured spectrophotometrically by the method of Giusti and Gakis [15]. The reaction started by the addition of the substrate (adenosine) to a final concentration of 21 mmol/L as previously described [7]**Erro! Fonte de referência não encontrada.**. The specific activity was reported as U/L.

2.1.4 Oxidative stress markers

Lipid peroxidation was determined by seric levels of thiobarbituric acid reactive substances (TBARS) according to Jentzsch et al. [16]. Results were obtained by spectrophotometry at 535 nm, and expressed in micromolar of malondialdehyde per milligram of protein (µmol of MAD/mg of protein). Mensuration of SOD and CAT activities were performed using whole blood collected into tubes with sodium citrate. CAT activity was

carried out in accordance with a modified method of Nelson and Kiesow [17], using an aliquot (0.02 mL) of blood (diluted 1:10 with saline) homogenized in 0.910 mL of 50 mM of 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 absorbance at 240 nm was measured for 2 min. SOD activity was based on the inhibition of O_2 when reacted to adrenalin [18]. A unit of SOD was defined as the amount of enzyme that inhibited 50% of the speed of the adrenalin oxidation. This reaction led to the formation of a red-colored product, adrenochrome, which was detected by a spectrophotometer at 480 nm, in a reaction medium containing 50 mM of glicine–NaOH, (pH 10) and 1 mM of adrenalin. CAT and SOD activities were calculated, and the results were expressed as nanomolar CAT per mg of protein (nmol CAT/mg of protein), and Unit SOD per milligram of protein (U SOD/mg of protein), respectively.

2.1.5 Statistical analysis

The data was firstly analyzed by descriptive statistics. Then, the data were tested for normality of variance by Shapiro-Wilk test, skewness, kurtosis and homogeneity by *Levene's* test and previously by Student's t-test. An unpaired (two-sample) t-test was used to compare both groups. It was considered significantly different when p-value was lower than .05. The statistical process was carried with R-language, v.3.3.0 (R Development Core Team, 2012).

2.2 Experiment II

2.2.1 Animals and samples

In this study, we analyzed 1,242 frozen serum samples of lactating dairy cows previously used to investigate leptospirosis [19]. These samples were obtained from adult cows of 69 herds of the Western part of Santa Catarina state (Figure 1). Out of these farms, 68 used semi-extensive production system, with a concentrate-based diet and free grazing, while

one farm used extensive system with confinement only during the lactation period. Among all surveyed herds, there were reports of reproductive problems in some animals, however, without a diagnosis for brucellosis.

2.2.2 Serology

Serology for brucellosis was performed using the buffered acidified antigen (BAA) test on all samples, and the 2-mercaptoethanol test for confirmation, as recommended by the Brazilian National Program for the Control and Eradication of Brucellosis and Tuberculosis (PNCEBT) [1, 20].

3. Results

3.1 Experiment I

The results of ADA activity are shown in Figure 2. The ADA was affected depending on the infections status of the animal ($t=6.36$, $p<0.001$), i.e. there was a decrease in the ADA activity in infected animals. TBARS and SOD results were different between groups, with an increase infected animals ($t=8.22$, $p<0.001$ and $t=6.34$, $p<0.001$, respectively) (Figure 3.A-B). Finally, CAT results showed a decrease in infected cows ($t=21.31$, $p<0.001$, Figure 2-C) compared to uninfected animals.

3.2 Experiment II

Among the animals surveyed, 331 (26.6%) had history of reproductive problems (repetition of estrus or abortion). However, none of the cows were seropositive for *B. abortus* (prevalence of 0%).

Discussion

A reduction in ADA activity in seropositive animals was verified in this study, which may reflect an increase in the concentration of extracellular adenosine. According to the literature, this reduction contributes to modulate the inflammatory response [21], since adenosine is an anti-inflammatory molecule, and, for this reason, when it is increased extracellularly it may help reducing the inflammation and, consequently, tissue damage [22]. A similar result was observed in an experimental infection by *Leptospira interrogans* serovar Icterohaemorrhagiae, in which ADA activity was reduced in serum and lymphocytes [23]. ADA is present in all mammalian tissues and it appears to play a role in the development and function of blood cells. It has been shown that problems in ADA activity of these cells are related to the development of some disorders [24]. The reduction in seric ADA activity observed can be related to the extracellular concentration of adenosine, which interacts with the purinergic receptor system and plays a protective role in vascular changes resulted from infection. Therefore, in brucellosis, as in other infectious diseases, ADA plays an anti-inflammatory role, helping to minimize the negative effects of an exacerbated inflammation.

The increase of TBARS in cows seropositives for *B. abortus* indicates the occurrence of lipid peroxidation, and consequently, damage to cell membranes allowing the reduction of the fluidity, and increase of extracellular permeability [4]. This contributes to the occurrence of oxidative stress, which is the result of an oxidative and antioxidant imbalance [25], observed in cows with brucellosis in the current study. It is well known that the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in several processes, such as phagocytosis, immunity and cellular defense, such as lipid peroxidation and aggression to the proteins of cells and membranes, to enzymes, carbohydrates and DNA [26, 27, 28]. Free radicals production is elevated in tissue damaged by infections due to a number of processes such as increased enzymes involved in radical formation, activation of

phagocytosis, iron and copper release, or an interruption of the electron transport chain [29]. If we evaluate each seropositive animal, it would be possible to verify that the highest levels of TBARS were present in three cows that had abortion, and therefore, it is possible to conclude that the oxidative stress can considerably contribute to the pathogenesis of the disease. According to the literature, the oxidant-induced endothelial damage can raise vascularization impairment and immunological malfunction, playing an important role in the pathophysiology of abortion [30]. Oxidative stress-induced placental dysfunction might be a common cause of multifactorial and polygenic etiologies of abortion with loss of recurrent gestation and defective embryogenesis [31]. These alterations may also occur in pregnant cows infected by *B. abortus* with an increase in the number of oxidative reactions observed in this study through oxidative stress markers such as lipid peroxidation, which was estimated by the quantification of TBARS in the serum and the activity of enzymatic antioxidants such as SOD and CAT in whole blood. However, it is important to note that this oxidation may negatively affect fertility, related to repeated oestrus described in cows with brucellosis. This can be explained by the fact that oxidant status of the cell modulates angiogenesis, which is critical for follicular growth, corpus luteum formation endometrial differentiation, and embryonic growth [30]. Since *B. abortus* is an intracellular gram-negative bacterium, it can escape from the immune system response, that determines its replication in the phagosome and the modulation of the immune response [32], which facilitates its dissemination, and ability to survive inside macrophages, and to inhibit the fusion of the phagosome with the lysosome, thus, avoiding its destruction by the reactive nitrogen and oxygen intermediates [33].

The antioxidant function assessed by the activity of SOD and CAT was different, since these enzymes are the first line of endogenous defense against neutralization of ROS. SOD is an enzyme that catalyzes the dismutation of the superoxide radical into hydrogen peroxide,

serving as the primary line of defense in response to oxidative stress [34], explaining why this enzyme showed high activity. The hydrogen peroxide formed by SOD is degraded by the action of CAT and it is able to cross the nuclear membrane and induce damage through enzymatic reactions [34]. In this study, CAT activity was lower in cows infected by *B. abortus*, which may have allowed oxidative stress to occur, but this lower activity could also be explained by the exhaustion of CAT, since this is the first antioxidant enzyme activated in an oxidative imbalance, always reducing after others are activated. According to the literature, oxidative stress has emerged like a promoter of several pregnancy-related disorders and increase offspring susceptibility to the disease, and this may occur by the impairment of antioxidant defense system and enhancement of ROS generation, which alter cellular signaling and/or damage cellular macromolecules [35]. Researchers say that antioxidants play an important role in maintaining gestation, and highlight the importance of antioxidant supplementation in female reproduction [30]. The need of antioxidant supplementation was indicated by the investigators of this case, however, it needs further investigation since inadequate supplementation can also cause damage.

In this study, we did not find cows with brucellosis in the Western part of Santa Catarina state, despite the surveillance and restrictions present in 150 herds. Previous studies performed in Santa Catarina state showed a prevalence of 0.2% and 0.6% [13, 36], and these data demonstrate that the state has historically a low prevalence of this disease, which may be a result of better structured official surveillance (test-and-slaughter policy), along with the profile of the farms (mainly small), as well as a lower movement of animals between properties and, mainly by the prohibition of the purchase of cattle for breeding from other Brazilian states (an imposition of the Ministry of Agriculture to maintain the state free of foot-and-mouth disease without vaccination) [13, 36]**Erro! Fonte de referência não encontrada.**. In other Brazilian states, the prevalence for brucellosis is higher, reaching up to 10.2% of the

cows in the state of Mato Grosso, Central Brazil [37]. Figure 1 shows the distribution of brucellosis in dairy cows in the Brazilian states published in scientific journals in the last 12 years (2004-2016). In South American countries, prevalence of *B. abortus* is also low, reaching 0.6% in Colombia [38], 3-4% in Paraguay [39], and 4-5% in Argentina [40], probably due to eradication programs.

In this study on brucellosis, it was possible to conclude: (1) pregnant cows seropositives for brucellosis suffer oxidative stress, which may enhance the occurrence of abortion; (2) the reduction in the ADA activity in seropositive cows is a compensatory mechanism to decrease the inflammatory process triggered by the disease and, consequently, the tissue damage that can lead to abortion; and (3) 1,242 cows from 69 herds in the Western part of Santa Catarina state are seronegatives for *B. abortus.*

Conflict of interest: The authors declare that they have no conflict of interest.

Ethics Committee: This study was approved by the Ethics Committee for Animal Experimentation of Universidade do Estado de Santa Catarina (CEUA/UDESC) under protocol number 7688080916.

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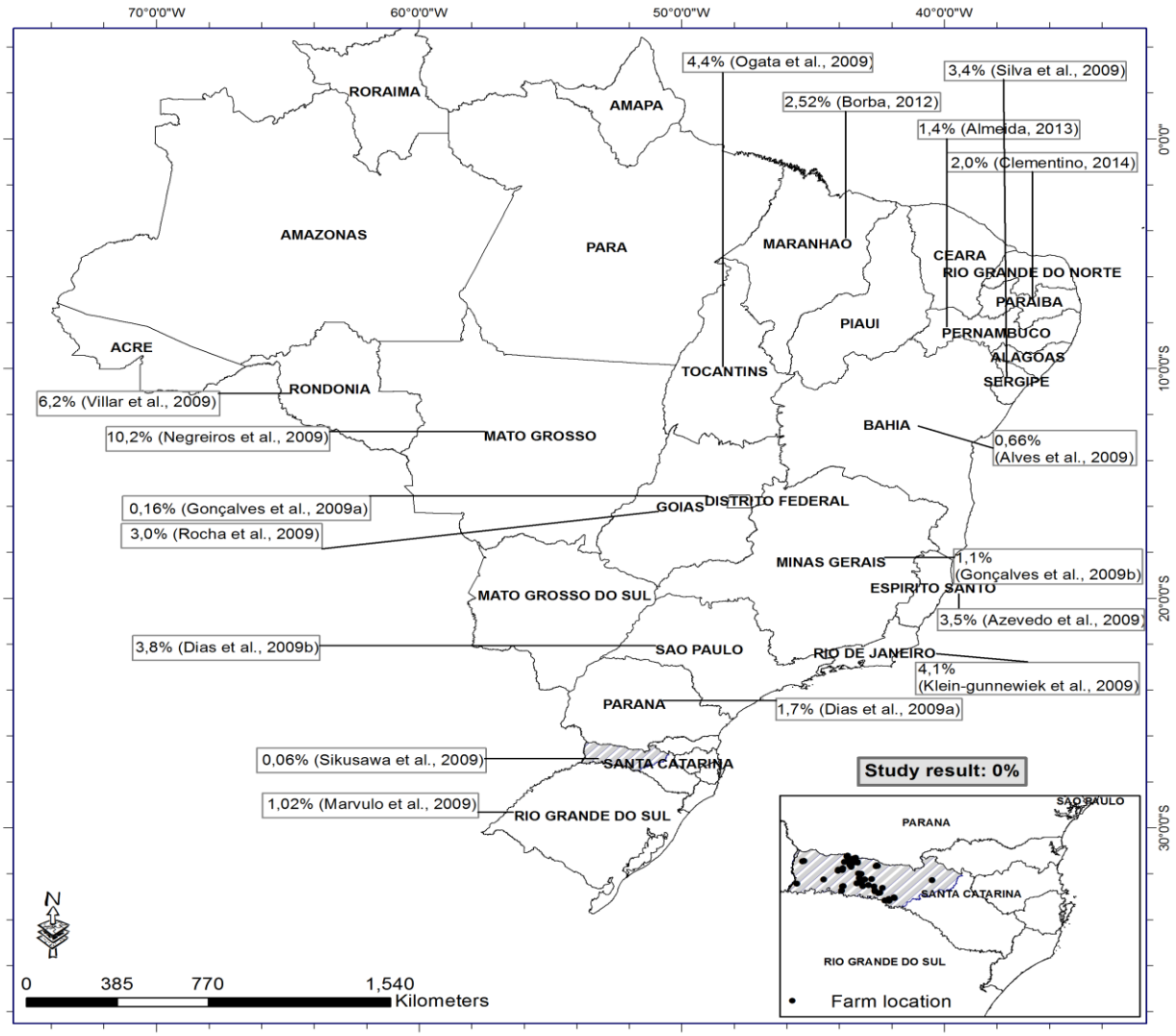

Figure 1: Experiment II – Map of the seroprevalence of *Brucella abortus* in cows in some Brazilian states in the last 12 years, as well as the studied region in the Western of Santa Catarina State, Brazil (black spots). Note: Marvulo et al. [41], Sikusawa et al. [13], Dias et al. [42], Klein-gunnewiek et al. [43], Dias et al. [44], Azevedo et al. [45], Gonçalves et al. [46], Rocha et al. [47], Gonçalves et al. [48], Negreiros et al. [37], Villar et al. [49], Ogata et al. [50], Borba [51], Silva et al. [52], Almeida [53], Clementino [54], Alves et al. [55].

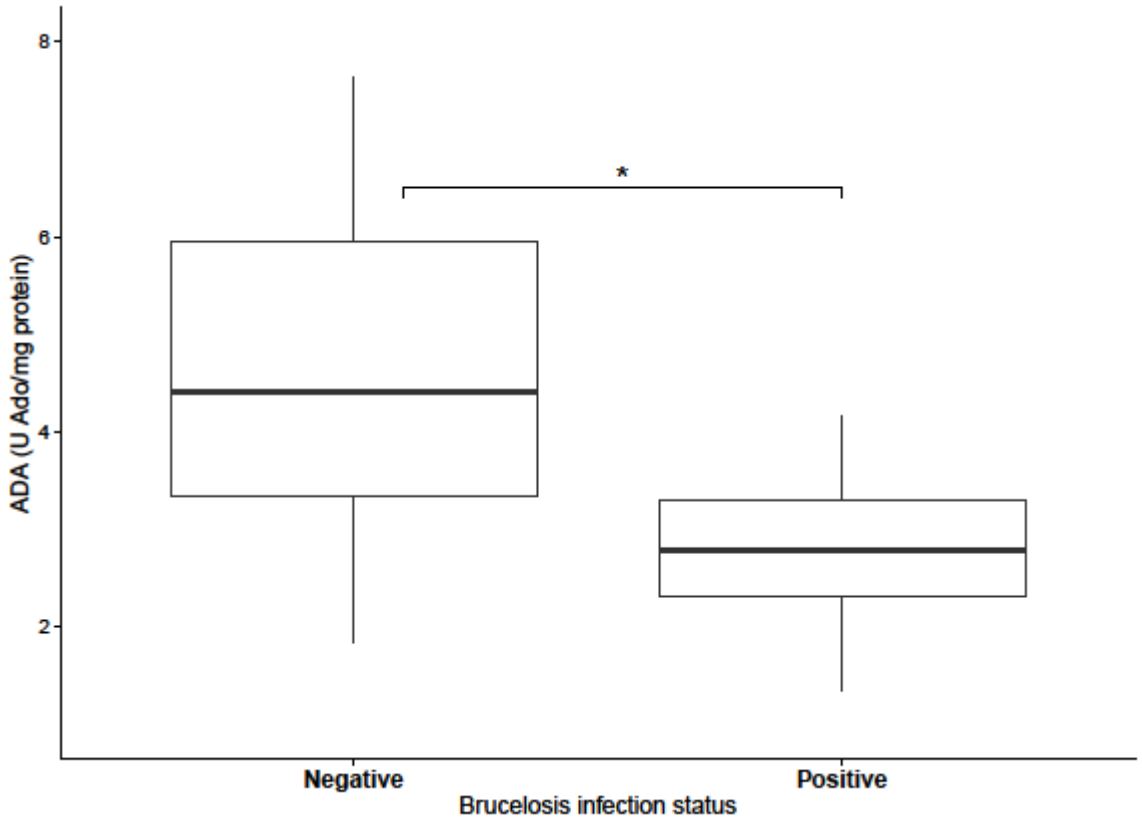

Figure 2: Experiment I - Activity of adenosine deaminase in the serum of negative cows (n = 10) in comparisons with positive cows (n = 10), median their quartiles. Activity of ADA differed ($p < 0.001$) from the animal's infection status.

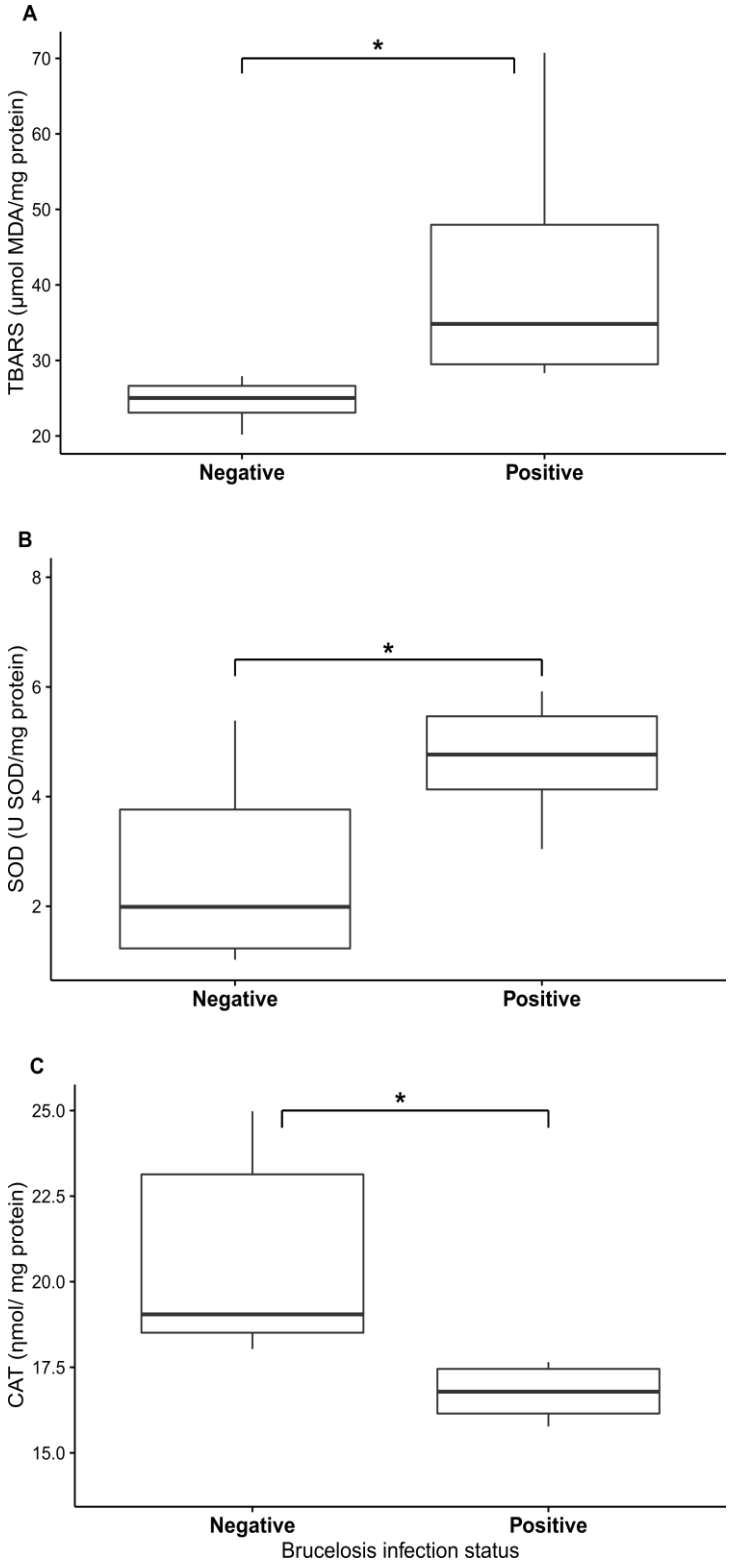

Figure 3: Level of TBARS in serum (A) and activities of SOD (B) and CAT (C) in whole blood from negative cows (n = 10) compared to positive cows (n = 10), median their quartiles. The levels of TBARS (t = 8.22), SOD (t = 6.34) and CAT (t = 21.31) differed ($p < 0.001$) among the groups in relation to the infection status of the animal.

2.2 – ARTIGO II

Hematological and biochemical disturbances caused by *Brucella ovis* infection using an experimental model

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Compliance with Ethical Standards. This experiment was approved by the Animal Welfare Committee of the State University of Santa Catarina (UDESC) under protocol number 0015317/2017.

Conflict of Interest: The authors declare that they have no conflict of interest.

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ABSTRACT. Ovine brucellosis is an infectious disease caused by *Brucella ovis,* a Gram-negative bacterium associated with high economic losses in consequence of fertility impairment and commercial restrictions. In this sense, it is important to emphasize that the pathological mechanisms of *B. ovis* infection are poorly understood, since it is recommended the euthanasia of affected animals. Thus, the aim of this study was to evaluate hematological and biochemical disturbances caused by this microorganism using mice as the experimental model. In this way, forty-eight animals were divided into two groups: uninfected (control) and infected. Hematocrit, leucogram, serum biochemistry (total protein, albumin, globulin and glucose), reactive oxygen species (ROS) and histopathology of liver and spleen were evaluated on days 7, 15, 30 and 60 post-infection (PI). Hematocrit of infected animals was lower than the control group on days 7, 15 and 30 PI, while leukocyte counts were higher as a consequence of increased lymphocytes, neutrophils and monocytes in some periods of the infection. Serum albumin levels were lower in infected animals compared to the control group in all evaluated periods, while serum glucose levels were lower on days 7 and 60 PI. On the other hand, serum globulin levels were higher in infected animals compared to the control group on days 15, 30 and 60 PI. Moreover, spleens of infected mice were severely enlarged, as well as increased levels of splenic and hepatic reactive oxygen species in various periods of infection as a Hematological and biochemical disturbances caused by *B. ovis* infection using a novel experimental model consequence of granulomatous splenitis and multifocal hepatic granuloma. Based on these results, we concluded that *B. ovis* infection can cause disorders related to the inflammatory response and exacerbation of free radicals linked to tissue lesions, which can be directly associated to a decrease in serum levels of albumin and glucose.

Keywords: brucellosis; disorders; hemogram; cell damage; histopathology.

INTRODUCTION

Ovine brucellosis is an infectious disease caused by an immobile, facultative and Gram-negative bacterium called *Brucella ovis* (Biberstein, 1964). This disease is characterized by genital lesions that lead to epididymitis and impairment of semen quality (Megid et al., 2010), and consequently, subfertility or infertility in males (Carvalho Junior et al., 2010), as well as mortality in female animals (Xavier et al., 2009). The transmission of the etiologic agent occurs mainly by ingestion or sexual contact with contaminated genital secretions (Alves et al., 2006), mainly semen (Paolicchi et al., 1993; Homse et al., 1995).

The inflammatory response mediated by *Brucella* genus results in evasion and suppression of the host immune response (Xavier et al., 2010; Antunes et al., 2013). Initially, the bacteria colonize the lymph nodes and reach the tissues of the genital tract after 30 days of infection (Nozaki et al., 2008). Bacteria belonging to *Brucella* genus use an interesting approach to be undetected by the host immune system, which includes a prolonged intracellular infection with genetic modification of the host, alterations of the apoptosis process and lower activation of protective mechanisms against inflammation (Rajashekara et al., 2006).This disease causes high economic losses and it has drawn attention worldwide due to the growth of sheep production and a lack of knowledge regarding the disease-causing agent (Lira and Megid, 2009). Moreover, knowledge regarding *B. ovis* infection on hematological and biochemical effects remains poorly understood. Thus, the aim of this study was to evaluate hematological and biochemical disturbances and correlate them to pathological findings using mice as experimental model for ovine brucellosis.

MATERIAL AND METHODS

Animals

Forty-eight male mice (*Mus musculus*, Swiss lineage; 60 days old; 30 ± 0.5 g) were used as experimental models. The animals were maintained in cages under controlled temperature and humidity (25 ºC, 70 %), fed with a commercial feed and water *ad libitum.* All animals were subjected to an environmental adaptation of 15 days.

Inoculum and experimental design

A virulent *B. ovis* strain (ATCC 25840), also known as NCTC10512 or 63/290, was used in this present study. The inoculum preparation was performed following the methodology described by Silva (2011).

The animals were divided into two groups of twenty-four animals each: uninfected mice (the control group) and *B. ovis* experimentally infected mice (100 µL containing 1.3 x 10^7 UFC/mL via intraperitoneal). The control group received 100 µL of phosphate buffer solution (PBS) intraperitoneally. All animals were observed daily.

Sample collection

Six animals from each group were euthanized on days 7, 15, 30 and 60 post-infection (PI) after anesthesia with isoflurane in an anesthetic chamber. A volume of 1000 µL of total blood was collected by cardiac puncture and divided into two tubes: one tube without anticoagulant and one tube containing EDTA as anticoagulant. The blood collected without anticoagulant was centrifuged at 3000 rpm for 10 min to obtain serum. The blood collected into tubes containing EDTA was used for enzymatic activities and hematocrit.

After blood collection, the animals were humanely euthanized by cervical dislocation. The presence or absence of macroscopic alterations was observed during the necropsy. The

spleens were weighted using an analytical balance (AY220-Marte) and the results were expressed in grams (g). Fragments of hepatic and splenic tissues were collected for measurement of free radical content and histopathological analyses.

Hematocrit and leucogram

Hematocrit (HT) was determined using the microhematocrit technique, as reported in details by Jain (1986). Leukocyte counts were determined using the hematological analyzer Celm (model CC530, Brazil). The differential leukocyte counts were performed in blood smears using the Romanowsky method followed by differential counts using optical microscopy, as recommended by Lucas and Jamroz (1961).

Serum biochemistry

Serum total protein, albumin and glucose levels were measured using commercial kits (Analisa®) and a semi-automatic biochemical analyzer BioPlus 2000. The globulin levels were obtained by the difference between total protein and albumin.

Splenic and hepatic reactive oxygen species (ROS) levels

ROS levels were determined by the DCFH oxidation method described by Ali et al. (1992) and recently published in details by Biazus et al. (2017). Fluorescence was measured using excitation and emission wavelengths of 485 nm and 538 nm, respectively. A calibration curve was established with standards of 2',7'-dichlorofluorescein (DCF) (0.1 nm to 1 μm), and results were expressed as U DCFH/mg of protein.

Histopathology

At the necropsy, tissue samples were collected from hepatic lobes and spleens, fixed in 10% buffered formalin, embedded in paraffin wax for hematoxylin and eosin (HE) staining and histopathological examinations were performed by a pathologist in a blinded fashion (Cardiff et al. 2014). Lesions were described and **sc**ored as followed: absent, mild, moderate, and severe.

Amplification of *B. ovis* DNA by PCR

In order to confirm *B. ovis* infection, a fragment of each spleen was collected and kept at -20 ºC. DNA extraction was performed using approximately 500 µL of macerated thawed spleen samples, as previously described (Matrone et al., 2009). For the amplification of *B. ovis* genomic DNA the protocol described by Xavier et al. (2010) was used. Briefly, primer pairs targeting the ORFs AO503 (F: 5´-GCCTACGCTGAAACTTGCTTTTG-3´ and R: 5´-ATCCCCCCATCACCATAACCGAAG-3´ and AO512 (F: 5´-TTCAGGCGACTGCTAATGGCAC-3´ and R: 5´-AAACCGATACCTCATCCCCGAG-3´) were used. Polimerase chain reaction (PCR) was performed using 23µL of a commercial PCR mix (Platinum PCR SuperMix, Invitrogen, Life Technologies, Carlsbad, EUA), 0.5µL of a 25µM solution of each primer, and 1–3 µL of template DNA (100–500 ng of DNA per reaction). Cycling parameters were denaturation (95 ºC for 5 min); 35 cycles of denaturation (95 ºC for 1 min), annealing (55 ºC for 1 min), and extension (72 ºC for 1 min); and a final extension (72 ºC for 5 min). PCR products were resolved by 1% agarose gel electrophoresis. Reactions were considered positives when they yielded products of 228 and 135 bp for primers targeting AO503 and A0512, respectively.

Statistical analyses

The data were subjected to the normality test (Shapiro-Wilk) and converted to logarithm when they did not show normal distribution. Later, the data were analyzed using Student's *t* test for independent samples considering significant when $P<0.05$. The results were showed as mean and standard deviation.

RESULTS

Clinical signs, such as apathy and bristled hair, were observed in experimentally infected mice in the beginning of the infection, i.e., on day 7 PI. Hematocrit content was reduced in animals experimentally infected by *B. ovis* on days 7, 15 and 30 PI compared to the control group, while total leukocyte counts were higher in the same days. Moreover, lymphocyte counts (day 7 PI), neutrophils (days 7, 15 and 30 PI) and monocytes (day 30 PI) were higher in the infected group compared to the uninfected control group (Table 1). Serum albumin levels were lower in mice experimentally infected by *B. ovis* on days 7, 15, 30 and 60 PI compared to the control group, while globulin levels were higher only on days 15, 30 and 60 PI. On the other hand, glucose levels were lower in experimentally infected animals on days 7 and 60 PI compared to the control group (Table 2).

As expected, no histopathological alterations were observed in spleen and liver tissues of control animals. Spleens of infected animals showed splenomegaly (Figure 1). Several pathological lesions were observed in splenic and hepatic tissues of animals experimentally infected by *B. ovis* as illustrated in Figure 2. On days 7 and 15 PI, it was noticed multifocal moderate granulomatous splenitis with epithelioid macrophages and rare neutrophils. In spleens, it was observed discrete to moderated nodular infiltration of epithelioid macrophages compatible to granuloma (day 30 PI) and moderated to severe multifocal and coalescent granulomatous splenitis adjacent to the pulp with epithelioid macrophages and rare neutrophils (day 60 PI). On day 7 PI, hepatic tissues showed discrete multifocal granuloma

with epithelioid macrophages and rare neutrophils, discrete multifocal necrosis with discrete inflammatory infiltrate, and moderate focal lymphohistiocytic infiltrate in adipose tissue adjacent to the liver. On day 15, discrete to moderate multifocal granuloma with epithelioid macrophages and rare neutrophils were observed in liver samples. On days 30 and 60, it was observed discrete to moderate random multifocal granuloma with epithelioid macrophages, as well as focally extensive area with moderate necrotic hepatocytes. Splenic ROS levels were higher in animals experimentally infected by *B. ovis* on days 7 and 30 PI compared to the control group, and it was also higher on days 7 and 15 PI in hepatic samples (Figure 3).

DNA fragments of *B. ovis* were not detected by PCR in spleens from uninfected mice. Considering infected animals, the specific PCR assay was able to detect *B. ovis* DNA in spleens.

DISCUSSION

In this study, mice experimentally infected by *B. ovis* showed a depressive behavior, apathy and bristled hair in the beginning of the infection (first seven days), which are common signs observed in the initial course of ovine brucellosis, in addition to fever, dyspnea and poor physical condition (Megid et al., 2010). Thus, mice can be considered a suitable experimental model to evaluate pathological mechanisms involved in *B. ovis* infection (Silva et al. 2011).

The mild reduction of hematocrit content on days 7, 15 and 30 PI can be associated to sequestration of erythrocytes in the spleen. *B. ovis* disseminates to the entire animal body, colonizing many tissues rich in cells of the phagocytic mononuclear system (Bathke, 1999), as well as may cause direct or indirect hemolysis. Another possibility, is that the immunodepression of the bone marrow caused by the disease leads to a minor release of circulating erythrocytes in mice, with reduced size, explaining the reduction of globular

volume. Also, human brucellosis is linked to the occurrence of granulomas in the bone marrow, contributing to occurrence of anemia (Correia, 2003).

Leukocytosis with increased neutrophils observed in mice *B. ovis* infected is characteristic of bacterial infections. This response occurs since the presence of bacteria stimulates the inflammatory response, as observed during infections caused by *Brucella* genus (Paulin, 2003; Lira and Megid, 2009). During pathological conditions, neutrophilia results in a release of medullar cells, including monocytes, which is common in chronic inflammatory conditions. Also, is important highlight that excessive neutrophils count can contribute to reduction of erythrocytes due its apoptotic action induced by proteolytic enzymes, as neutrophil serine proteases, that participates in the pathogenesis of inflammatory diseases (Kessenbrock et al., 2011).

Serum biochemistry alterations associated with hyperglobulinemia, hypoalbuminemia and hypoglycemia were observed in mice infected by *B. ovis*, as observed by Greene and George (1984) during the chronic phase of disease in dogs. The antigenic stimulus elicited by bacteria lead to a humoral response with excessive globulin production, which increases blood viscosity, and consequently, hyperglobulinemia. The augmentation of blood lymphocytes on day 7 PI suggests an intense inflammatory response, which can be stimulated the release of immunoglobulins or acute-phase proteins, reflecting to increased serum globulins levels. The reduction of albumin must be directly related to the hepatic lesions caused by the infection, that is to say, due to the decrease in organ function, consequently less albumin synthesis occurs, as reported by Santarém et al. (2008) in an infection caused by *Ehrlichia canis* in dogs and by Da Silva et al. (2010) during *Trypanosoma evansi* infection.

The results cited above suggest that hypoalbuminemia can be a secondary cause of hyperglobulinemia, acting as a compensatory mechanism in order to maintain the plasma osmolarity, since the serum total protein levels were unaltered probably due to these effect on

globulins (Kanavamal et al., 2002). Hypoglycemia was observed on infected animals 7 days PI, which can be related to apathy in the same period of infection. Also, this reduction can be associated to consumption of glucose by bacteria during the acute phase of disease.

Anatomopathological alterations were verified in infected mice, such as splenomegaly mainly in the initiation of infection, which can be linked to high bacterial counts in the acute phase of infection. Inflammatory and anatomopathological alterations as multifocal granulomas, among others, have been described in mice infected by *B. ovis* (Silva et al. 2011), as also described in brucellosis by Campos et al. (2009) and Matrone et al. 2009). In this study, we observed a significantly increase on splenic and hepatic ROS levels in animals experimentally infected, as a direct consequence of tissue damage in these organs. This molecule is associated to oxidative, inflammatory and deleterious effects when in excess (Barreiros et al., 2005), contributing to tissue lesions observed in this present study, as observed by Baldissera et al. (2014) in the liver and kidney of rats experimentally infected by *T. evansi.* Also, excessive ROS content may be linked with reduction of erythrocyte count, since the oxidative ROS effects interacts with membrane of erythrocytes and induce apoptosis, as observed by Hattangadi and Lodish (2007). Thus, the augmentation of ROS content can be a pathway linked with reduction of erythrocyte count during brucellosis.

CONCLUSION

B. ovis infection using mice as an experimental model elicited a reduction on hematocrit content in the acute phase of infection, as well as an exacerbated inflammatory response, which can contribute to splenic and hepatic tissue damage. In addition, the hepatic tissue damage can also contribute to alterations on hepatic function, as observed in this study by decreased serum albumin levels. Finally, the augmentation of splenic and hepatic ROS levels may direct contribute to tissue damage observed in histopathological analyzes.

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Table 1: Hematological analyzes of mice experimentally infected by *Brucella ovis.*

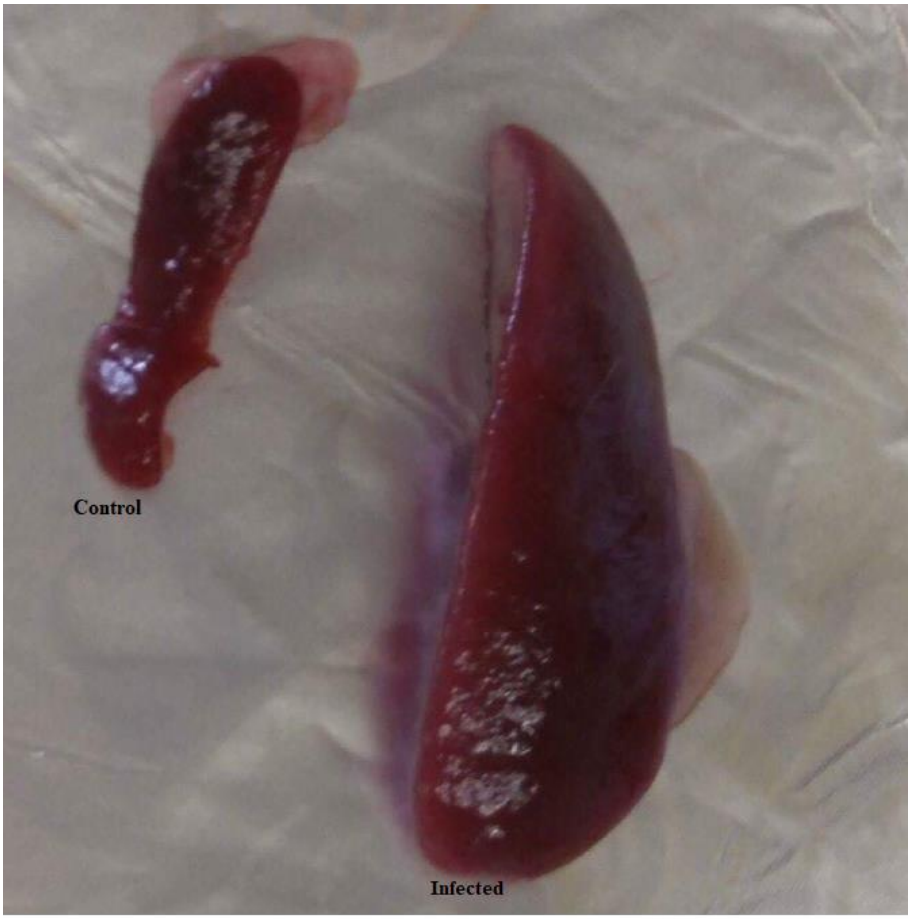
Variable	Sampling day	Control	Infected	P value
Hematocrit (%)	7	39.5 ± 3.56	31.3 ± 2.33	0.001
	15	35.8 ± 2.13	28.1 ± 2.78	0.001
	30	41.3 ± 3.44	36.5 ± 4.7	0.042
	60	38.0 ± 6.0	37.0 ± 3.10	0.870
Total leukocytes (µL)	7	2040 ± 260.7	3166.6 ± 715.5	0.001
	15	1733.3 ± 240.8	2783.3 ± 552.2	0.001
	30	2360 ± 194.9	3240 ± 680.3	0.022
	60	2020 ± 571.8	2340 ± 279.2	0.454
Lymphocytes (µL)	7	1438.4 ± 232.6	2230.3 ± 707.3	0.030
	15	1326.0 ± 319.9	1498.0 ± 331.6	0.758
	30	1823.2 ±116.9	1781.6 ± 785.6	0.644
	60	1577.2 ± 580	1739.2 ± 397.5	0.717
Neutrophils (µL)	7	457.6 ± 216.5	784.3 ± 116.3	0.012
	15	345.0 ± 98.3	1107.9 ± 392.5	0.001
	30	453.2 ± 216.2	1250 ± 605.4	0.001
	60	382.8 ± 165	432.4 ± 236.5	0.586
Monocytes (µL)	7	99.2 ± 68.7	100.6 ± 80.6	0.457
	15	28.6 ± 15.23	107.6 ± 122.0	0.244
	30	56.4 ± 20.2	178.4 ± 77.3	0.039
	60	13.3 ± 20.4	121.6 ± 52.6	0.010
Eosinophils (µL)	7	25.6 ± 23.4	32.0 ± 85.8	0.651
	15	28.6 ± 39.3	69.8 ± 128.1	0.537
	30	17.6 ± 39.2	49.2 ± 48.0	0.498
	60	37.6 ± 28.4	46.8 ± 5.5	0.354
Basophiles (µL)	7	48.8 ± 36.0	8.6 ± 23.2	0.187
	15	5.0 ± 13.4	0.0 ± 0.0	0.819
	30	9.2 ± 20.5	0.0 ± 0.0	0.743
	60	8.8 ± 19.6	0.0 ± 0.0	0.753

Difference between groups when $P<0.05$.

Table 2: Serum biochemical parameters of mice uninfected and experimentally infected by *Brucella ovis.*

Variable	Day of sample	Control	Infected	P value
Total proteins (mg/dL)	7	6.74 ± 0.97	7.38 ± 0.85	0.47
	15	5.52 ± 1.0	6.08 ± 0.41	0.36
	30	6.73 ± 0.53	6.93 ± 1.28	0.76
	60	6.36 ± 0.57	6.44 ± 0.43	0.84
Albumin (mg/dL)	7	2.12 ± 0.23	1.60 ± 0.33	0.04
	15	2.26 ± 0.21	1.68 ± 0.12	0.00
	30	3.05 ± 0.50	2.16 ± 0.27	0.00
	60	3.18 ± 0.27	2.3 ± 0.14	0.00
Globulin (mg/dL)	7	4.62 ± 0.77	5.38 ± 0.85	0.09
	15	3.26 ± 1.06	4.40 ± 0.50	0.04
	30	3.68 ± 0.75	4.76 ± 0.86	0.01
	60	3.18 ± 0.41	4.14 ± 0.40	0.00
Glucose (mg/dL)	7	307.8 ± 77.5	222.8 ± 49.4	0.01
	15	215.2 ± 52.9	220.8 ± 39.5	0.69
	30	287.0 ± 45.9	240 ± 71.1	0.21
	60	279.8 ± 29.5	233.0 ± 43.5	0.04

Difference between groups when $P<0.05$.



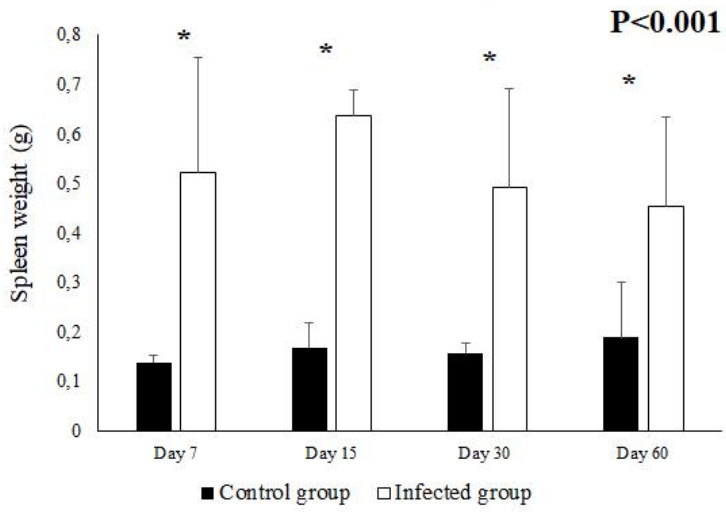


Figure 1: Spleen of uninfected (the control group) mouse and from a mouse experimentally infected by *Brucella ovis.* The graphic shows the difference between groups regarding spleen´s weight.

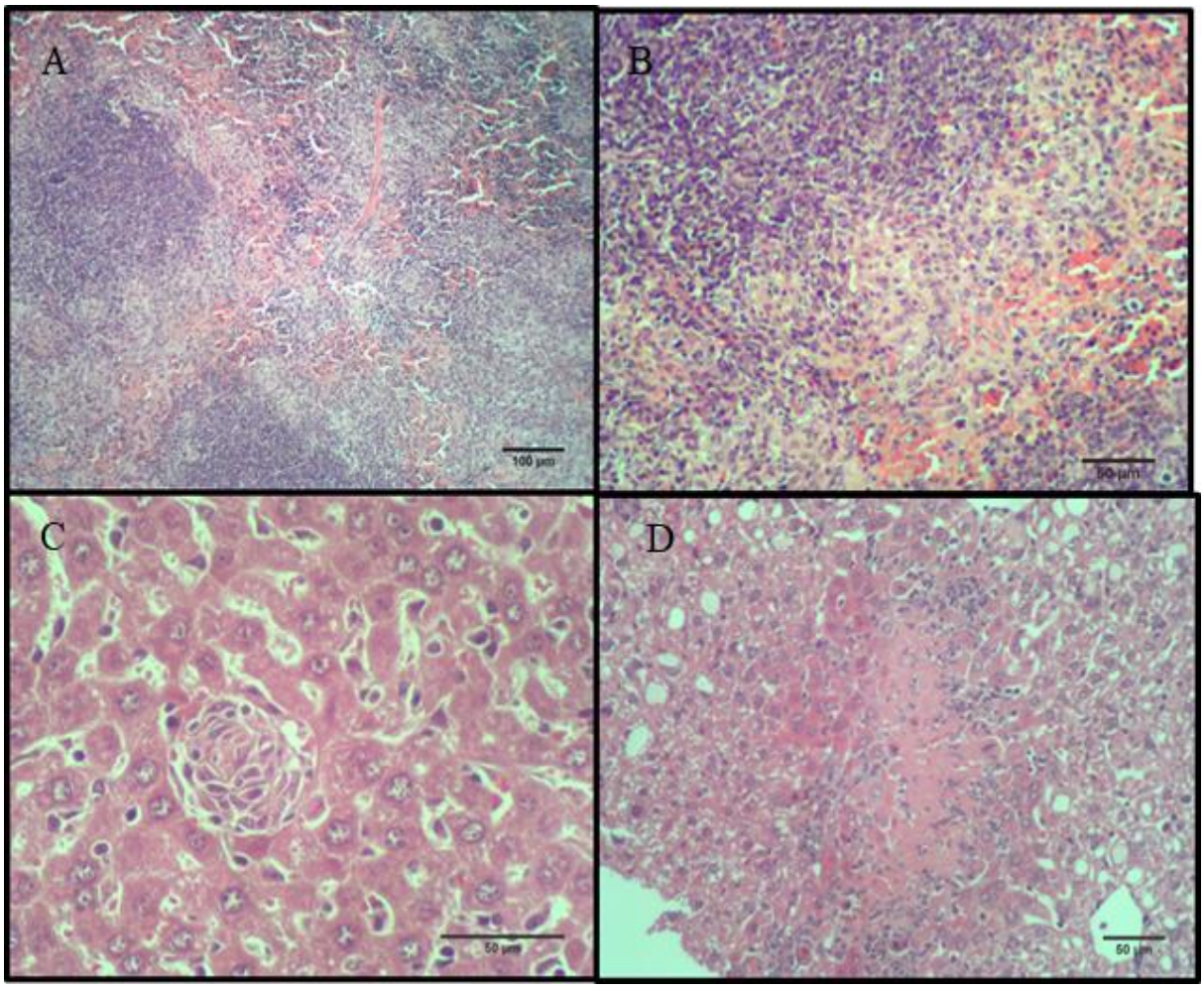


Figure 2: Histopathology of mice experimentally infected by *Brucella ovis* in different periods of infection. (A) Spleen: accentuated granulomatous splenitis (*) multifocal to coalescent on day 60 post-infection (PI) (HE, 100 x). (B) Spleen: Granuloma detail (*) adjacent to the pulp on day 60 PI (HE, 200 x). (C) Liver: multifocal granuloma on day 30 PI (HE, 400 x). (D) Liver: necrotic central area on day 60 PI (HE, 200 x).

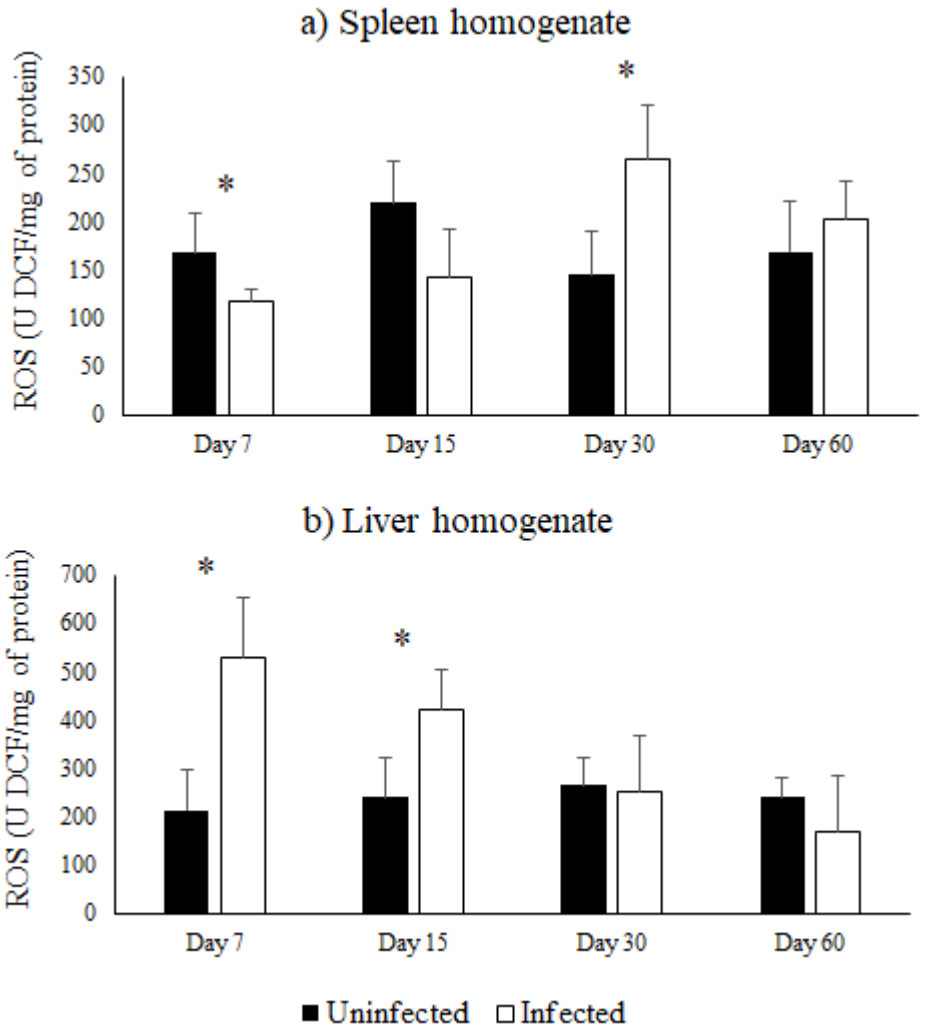


Figure 3: Splenic [A] and hepatic [B] reactive oxygen species (ROS) levels of mice experimentally infected by *Brucella ovis* on days 7, 15, 30 and 60 post-infection (PI). (*P<0.05).

2.3 – ARTIGO III

Adenosine deaminase behavior in experimental infection by *Brucella ovis* and its participation in the modulation of the inflammatory response

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Adenosine deaminase behavior in experimental infection by *Brucella ovis* and its participation in the modulation of the inflammatory response

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Compliance with Ethical Standards. This experiment was approved by the Animal Welfare Committee of the State University of Santa Catarina (UDESC) under protocol number 0015317/2017.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Abstract

The objective of this study was to evaluate the activity of adenosine deaminase (ADA) and its participation in the modulation of the inflammatory responses of mice experimentally infected with *B. ovis*. We used 48 animals, divided in two groups: group A (control) and group B (infected). We evaluated ADA activity and C-reactive protein (CRP) concentrations at four time points (days 7, 15, 30 and 60) post-infection (PI). There was a reduction in ADA activity in the acute phase (day 15 PI), followed by elevation when the infection became chronic (days 30 and 60 PI). Serum C-reactive protein levels were higher in infected animals than in the control group at all-time points post-infection ($P < 0.05$). ADA regulates levels of extracellular adenosine, an anti-inflammatory molecule, suggesting that the enzyme had different behaviors depending on the stage of infection, i.e., ADA had anti-inflammatory action in the acute phase, but with the chronicity of the disease the ADA had pro-inflammatory action. Therefore, we conclude that ADA participates in the pathogenesis of experimental ovine brucellosis.

Keywords: brucellosis; adenosine deaminase; inflammation; pathogenesis.

Introduction

Ovine brucellosis by *Brucella ovis* is an infectious-contagious cosmopolitan disease (Lira and Megid 2009). In general, even in Brazil, studies of *B. ovis* infection have been scarce, despite the economic losses associated lower herd fertility. Furthermore, when brucellosis appears in herds, there is consequent limitation in the marketing of products and by-products, as well as in breeding (Jahans et al. 1997). Early diagnosis is an alternative to reduction or eradication of the disease, since brucellosis has no specific treatment (Martins et al. 2012).

One of the consequences of brucellosis by *Brucella* spp. is infertility in males and abortion in females. There is a series of biochemical events that, if exacerbated, may be directly related to reproductive losses, as is the case when oxidative stress is evident in cattle with brucellosis (Perin et al., 2017). To minimize deleterious effects, a number of enzymatic responses occur that modulate inflammation and reduce cellular and tissue damage. Among these enzymes is adenosine deaminase (ADA), an enzyme responsible for the regulation of extracellular adenosine (Sauer et al., 2012) that possesses anti-inflammatory (Antonioli et al., 2012) and neuromodulatory (Wei et al. 2011) properties.

According to the literature, ADA is involved in various biological functions, and it is important to note that its anti-inflammatory role has been highlighted in infectious diseases such as Chagas disease (Da Silva et al. 2011), leptospirosis (Tonin et al. 2012), sporotrichosis (Castro et al. 2012), cryptococcosis (Azevedo et al. 2014), salmonellosis (Boiago et al. 2016), as well as in brucellosis caused by *Brucella abortus* (Perin et al. 2017). In cows naturally infected with *B. abortus* (Perin et al. 2017), ADA activity was lower than in non-infected cows, classified by the authors as an anti-inflammatory effect of ADA. Thus, in *B. ovis* infection, ADA activity may change in response to inflammation and thus may influence the pathogenesis of the disease. Therefore, the objective of this study was to evaluate serum ADA activity in mice experimentally infected by *B. ovis* and to study their effects on inflammatory responses to infection.

Materials and methods

Forty-eight mouse serum samples were used, kept frozen until analysis. The samples came from mice in two groups: non-infected (control; n=24) and infected by *B. ovis* (infected; n=24) collected at four post-infection time points (PI) (days 7, 15, 30 and 60), according to the publication of Perin et al. (2018). The animals were experimentally infected with *B. ovis*

(100 µL containing 1.3 x 10^7 UFC/mL intraperitoneally) and infection was confirmed by spleen PCR (Perin et al. 2018).

Serum ADA activity was measured spectrophotometrically by the method of Giusti and Gakis (1971). The reaction started by the addition of the substrate (adenosine) to a final concentration of 21 mmol/L, and incubation was carried out for 1 h at 37 °C. The reaction was stopped by the addition of 106 mmol/L/0.16 mmol/L phenol-nitroprusside/mL solution. The reaction mixture was immediately mixed with 125 mmol/L/11 mmol/L alkaline-hypochlorite (sodium hypochlorite) and vortexed. Ammonium sulfate (75 µmol/L) was used as the ammonium standard. The concentration of ammonia was directly proportional to the absorption of indophenol at 650 nm.

The quantification of serum C-reactive protein (CRP) was performed using a commercial kit for ultrasensitive CRP (BioTécnica, Minas Gerais, Brazil) following the manufacturer´s protocol on a Bio-2000 semiautomatic analyzer.

The data were first subjected to a normality test. As they did not present normality, they were transformed to logarithmic form. The comparison between means were performed by the student test (t-test). Differences between groups were considered significant when $P < 0.05$. The results were presented as means and standard deviations.

Results and discussion

ADA activity results are shown in Figure 1. Serum ADA activity was lower on day 15 PI in the animals in the *B. ovis* infected group than in uninfected mice ($P < 0.05$). Subsequently, an increase in activity was observed on days 30 and 60 PI ($P < 0.05$). Serum C-reactive protein (CRP) levels were higher ($P < 0.05$) in infected animals than in the control group at all times of infection (Fig. 2). CRP is one of the acute phase proteins, used as an inflammatory marker for various diseases, which can have its serum concentration increases

or decreases by at least 25% during the inflammatory response (Aguiar et al. 2013). Therefore, the mice infected with *B. ovis* developed an inflammatory response, which was maintained throughout the experimental period.

In mice infected with *B. ovis*, lower ADA activity was observed in the acute phase (Day 15), possibly reflect an increase in extracellular adenosine concentration, as previously described in *B. abortus* infections (Perin et al., 2017). Thus, ADA participates in the modulation of the inflammatory response (Abbrachio and Ceruti 2007), as it regulates levels of adenosine, an anti-inflammatory molecule that when released in the extracellular space may contribute to the reduction of inflammation and, consequently, tissue damage (Xaus et al. 1999). In addition, adenosine plays a protective role in vascular changes resulting from infection when interacting with the purinergic receptor system (Drury and Szent-Gyorgyi, 1929), an effect that is beneficial to animal health. As described for other infectious diseases (see the Introduction section of this manuscript), in ovine brucellosis, ADA has an anti-inflammatory role in the acute phase up to 15 days PI, therefore, an alternative route may attenuate the exacerbated inflammatory response, with negative consequences for the host.

When *B. ovis* infection became chronic (Days 30 and 60 PI), there was an increase in ADA activity, suggesting a decrease in adenosine levels in the extracellular environment and consequently a pro-inflammatory response of ADA, aiming to reinforce the inflammatory response in an attempt to combat the proliferation of the etiological agent (Burnstock 2006; Desrosiers et al.,2007). Throughout the experiment, there were neutrophil-mediated inflammatory responses and an increase in globulins (Perin et al., 2018), confirmed in this study by the increase of the C-reactive protein (CRP), a non-specific acute phase reactant produced by the liver in response to inflammatory stimuli; it is a highly-sensitive marker of inflammation, infection and tissue damage (Lind 2003; Suleiman et al. 2006). Serum levels of CRP are elevated by several factors during inflammation and tissue injury due to the fact that

its primary function is related to opsonization of pathogens by activation of the classical pathway of complement and modulation of the action of monocytes and macrophages (Eckersall, 2010; Petersen et al. 2004), presumably the same mechanism that occurred during *B. ovis* infection in an attempt to attenuate the infection.

Mice experimentally infected by *B. ovis* developed an inflammatory response, confirmed by increased CRP. ADA activity differed during *B. ovis* infection. In the acute phase, its activity was reduced, but as the disease became chronic, activity increased in mice. We observed that the enzyme had different behavior depending on the stage of infection: ADA had anti-inflammatory action in the acute phase and pro-inflammatory action in the chronic phase the disease. Therefore, we conclude that ADA participates in the pathogenesis of experimental ovine brucellosis in the experimental model used, mice.

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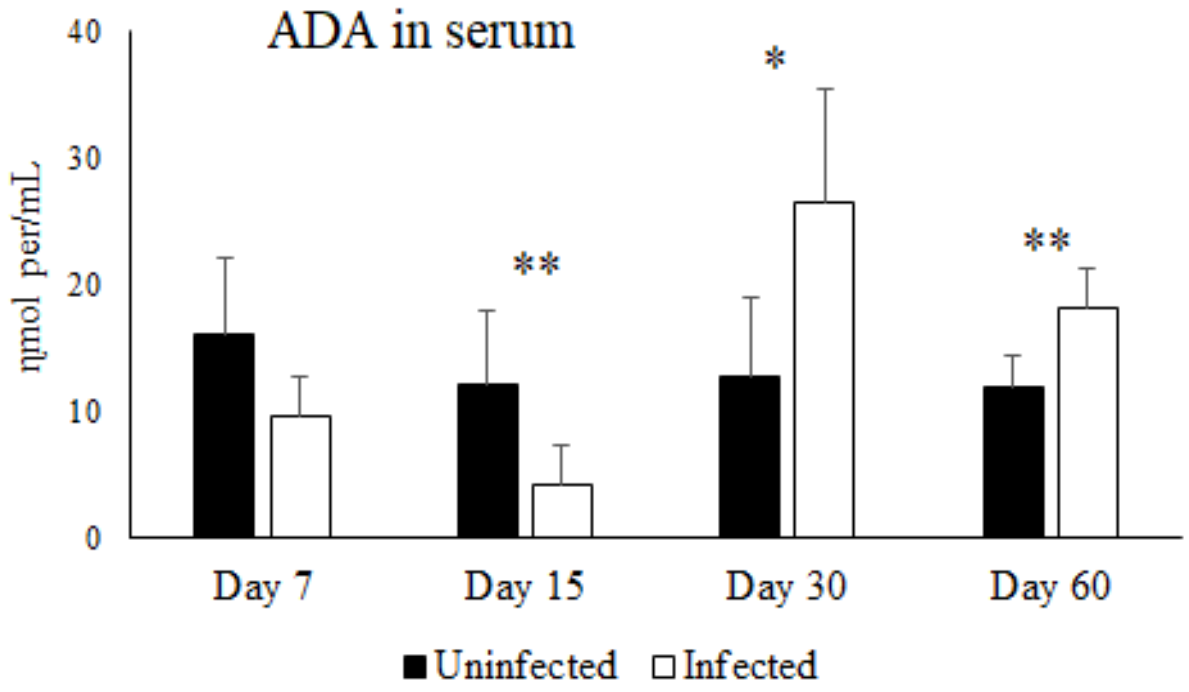

Figure 1: Serum adenosine deaminase (ADA) deaminase. Analysis performed in mice experimentally infected with *B. ovis*, measured on days 7, 15, 30 and 60 post-infection (*P<0.05 **P<0.001). Results are expressed as mean ± standard deviation.

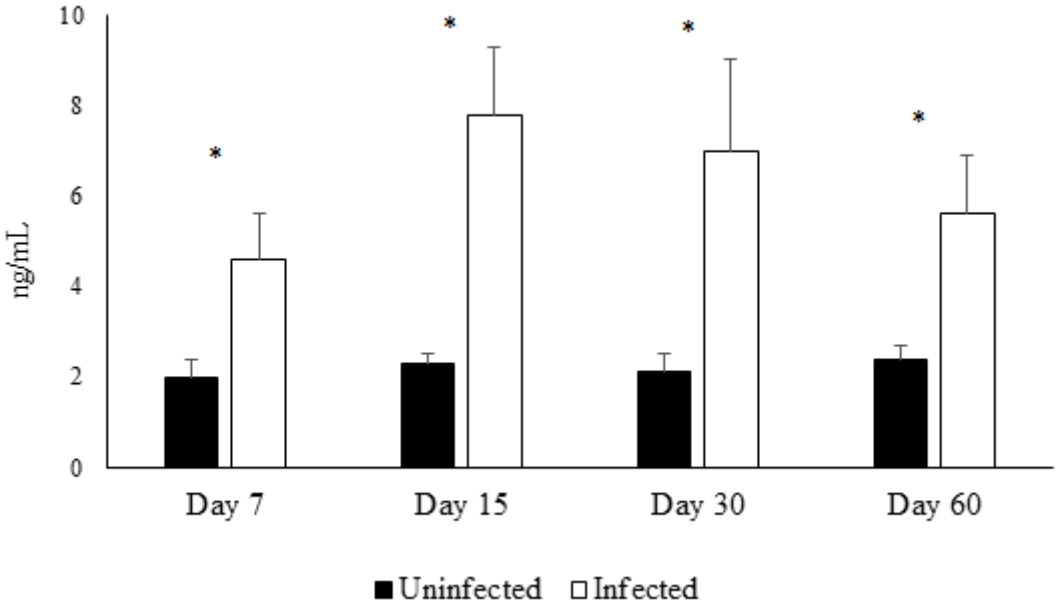

Figure 2: Serum concentrations of C-reactive protein (CRP) in mice experimentally infected with *B. ovis*, measured on days 7, 15, 30 and 60 post-infection (*P <0.05). Results are expressed as mean ± standard deviation.

2.4 – MANUSCRITO I

Experimental infection by *Brucella ovis* alters the activity of cholinesterases in blood and brain: inflammatory response and neuromodulation

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ABSTRACT

Purpose. The role of cholinesterases in inflammatory reactions has been described in several infectious diseases. However, in *Brucella* spp. this has not yet been studied. Therefore, the objective of this study was to evaluate whether experimental infection by *Brucella ovis* alters the cholinergic activity in pro- or anti-inflammatory responses to the disease.

Methodology. We used 48 mice, 24 infected by *B. ovis* and 24 non-infected. We collected samples of whole blood and brain on days 7, 15, 30 and 60 post-infection (PI) by *B. ovis*.

Results. AChE activity in the blood increased on days 15 and 30 PI (P <0.05), as well as in brain homogenates on day 60 PI (P <0.05). Butyrylcholinesterase (BChE) activity in serum also increased on days 7 and 60 PI (P <0.05). An increase in serum free radical levels occurred on days 7, 15 and 60 PI (P <0.05), and consequently superoxide dismutase activity increased on day 15 PI (P <0.05). A reduction in catalase activity occurred when the infection became chronic (60 PI).

Conclusions. The increase in AChE and BChE characterized a pro-inflammatory response, since these enzymes regulate levels of acetylcholine (ACh), a molecule with anti-inflammatory properties. Therefore, with the increase of cholinesterase activity, there was an extracellular reduction of ACh, an inhibitor of several inflammatory mediators. This proinflammatory response of *B. ovis* infection leads to oxidative stress, and consequently to cellular damage. In the brain, increased AChE may reduce levels of ACh which is also an important neurotransmitter, thus interfering with neurotransmission.

Keywords: Ovine brucellosis; acetylcholinesterase; butyrylcholinesterase; oxidative stress.

Abbreviations: PI, post-infection; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ACh, acetylcholine, TNF, tumor necrosis factor; IL-6, interleukin-6; IL-18, interleukin-18; IL-10, interleukin-10; EDTA, ethylenediaminetetraacetic acid; DTNB, dithiobisnitrobenzoic acid; AcSCh, acetylthiocholine iodide; BuSCh, butyrylthiocholine; ROS, reactive oxygen species; DCFH, dichlorofluorescein; CAT, catalase; SOD, superoxide dismutase.

INTRODUCTION

Brucellosis by *Brucella ovis* causes epididymitis and is therefore a major cause of infertility in sheep [1]; Nevertheless, the bacterium can be found in several organs. Studies on ovine brucellosis remain scarce and focus primarily on the pathogenesis of the infection caused by *B. ovis* [2]. According to the literature, the genus *Brucella* induces only a moderate inflammatory response, likely as a result of its evasion and suppression of the immune response in the susceptible host [3,4]. It is known that the inflammatory response involves several mechanisms, some of which are traditional, involving lymphocytes, cytokines and nitric oxide, and others more recently described, including the cholinergic system [5].

In most pathologies, cholinergic signaling mediates an anti-inflammatory response [6], regulated by cholinesterases. This is because acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are responsible for hydrolyzing acetylcholine (ACh) [7] during cholinergic signaling [8], assuming an important role in the host immune response [9]. ACh, in turn, attenuates the release of pro-inflammatory cytokines [10], including tumor necrosis factor (TNF), interleukin-6 (IL-6) and interleukin-18 (IL-18) without affecting the production of anti-inflammatory cytokines such as interleukin-10 (IL-10) [11]. Our hypothesis was that cholinesterase activity modulated ACh levels during *Brucella* spp. infection in order to minimize the deleterious effects of the disease. Therefore, the objective of this study was to evaluate whether the experimental infection with *B. ovis* altered cholinergic activity in pro- or

anti-inflammatory responses to the disease.

MATERIALS AND METHODS

Animals and samples

Serum, whole blood and brain samples of 48 mice from a previously published study [12] were used for this study. The material was collected on days 7, 15, 30 and 60 post-infection (PI) by *B. ovis*. The experimental design included two groups: non-infected mice (control: n = 24) and *B. ovis* infected group (n = 24). Six animals from each group were used at each collection time. A volume of 100 µL containing 1.3 x 10^7 CFU/mL was used to infect mice via intraperitoneal injection. The infection was confirmed by PCR of bacterial DNA detected in the spleen at the fourth collection time point during the experiment [12]. Serum was obtained by centrifugation of blood collected and allotted without anticoagulant and centrifuged (5500 g for 10 min) and frozen (-20°C). Blood was collected in all cases with anticoagulants EDTA and sodium citrate for analysis of AChE and antioxidant enzymes, respectively. The brains were kept frozen (-20°C) until analysis.

1.2.4 Acetylcholinesterase (AChE) activity in whole blood and brain

Samples with anticoagulant (EDTA) were diluted 1:50 (v/v) in lysis solution (0.1 mmol/L potassium/sodium phosphate buffer containing 0.03% Triton X-100) to determine AChE activity in blood samples, and were frozen at -20ºC for seven days. The AChE (EC 3.1.1.7; AChE) enzymatic assay was determined in whole blood by a method previously described [13, 14]. The activity was obtained using acetylcholine (ACh) as substrate and the enzymatic activity was expressed as µmol AcSCh/h/µmol of hemoglobin.

AChE activity was measured in brains [13] as previously described [15]. Samples from brain were rinsed in ice-cold saline and were homogenized to obtain 10% homogenate in

phosphate buffer (0.1 M, pH 7.4). The homogenates were centrifuged at 9000 x g at 4ºC for 20 min. The supernatants were stored at −30 ºC until analysis. Brain protein was adjusted for analysis between 1.4 – 1.8 μg/mL. AChE in brain involved a reaction mixture (330 μL final volume) containing 100 mM K^+-phosphate buffer, pH 7.5 and 1 mM 5,5′-dithiobisnitrobenzoic acid (DTNB). Both methods are based on the formation of the yellow anion, 5,5′-dithio-bis-acid-nitrobenzoic, measured by absorbance at 412 nm during 2-min incubation at 25°C. The enzyme (40–50 μg of protein brain) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). All samples were run in duplicate or triplicate and the enzyme activity was expressed in μmol AcSCh/h/mg protein.

Butyrylcholinesterase (BChE) activity in serum

Serum BChE (EC 3.1.1.8; BUChE) activity was determined as described [13]. BChE activity was assayed in a medium containing sodium phosphate buffer 0.1 mM, pH 7.4, DTNB 0.30 mM and 15 μL of serum. After 3 min of pre-incubation at 37°C, the reaction was started with butyrylthiocholine (BuSCh) 1 mM and reading was performed for 2 min at intervals of 20–20 s on a spectrophotometer at 412 nm. The specific activity was expressed in μmol of BcSCh hydrolyzed/h/mg of protein.

Reactive oxygen species (ROS) levels

Determination in serum of ROS was evaluated by 2´-7´-dichlorofluorescein (DCFH) levels as an index of peroxide production by cellular components. This experimental method is based on the deacetylation of the probe DCFH-DA, and its subsequent oxidation by reactive species to DCFH, a highly fluorescent compound [16]. Serum samples were added to a medium containing Tris–HCl buffer (10 mM; pH 7.4) and DCFH-DA (1 mM). After

DCFH-DA addition, the medium was incubated in the dark for 1 h until the start of fluorescence measurement procedure (excitation at 488 nm and emission at 525 nm, and both slit widths were 1.5 nm). The results ROS were expressed by U DCF/μL.

Catalase and superoxide dismutase activity

CAT (EC 1.11.1.6) activity was determined in blood by the decomposition of H_2O_2 at 240 nm according method described [17] and modified [18]. Superoxide dismutase (EC 1.15.1.1) activity was quantified [19] by spectrophotometrically determining the inhibition of auto-oxidation of epinephrine to adrenochrome at an alkaline pH at 480 nm in blood, liver and muscle. For the SOD assay, 10–40 μg of protein was used, and CAT activity was determined using 10–40 μg protein. The results were expressed in ηmoles/mg protein.

Protein determination

Protein was determined by the Coomassie blue method according as described [20] using bovine serum albumin as standard.

Statistical analysis

The data were subjected to the normality test. As there was no normality, data were transformed to logarithmic form. Then comparisons between means were analyzed by the student test (t-test) was performed. Significant difference between groups was assumed when $P < 0.05$. The results were presented as mean and standard deviation.

RESULTS

AChE activity in blood increased on days 15 and 30 PI in infected animals (Fig. 1a), as well as brain on day 60 PI ($P < 0.05$) compared to control (Fig. 1b). Serum BChE activity was also higher in the infected animals on days 7 and 60 PI ($P < 0.05$) compared to the uninfected animals (Fig. 2).

We observed increases in ROS levels in serum of the animals of the group infected with *B. ovis* on days 7, 15 and 60 post infection ($P < 0.05$; Fig. 3). SOD activity differed between groups only on day 15 PI ($P < 0.05$), when it was higher in infected animals (Fig. 3). CAT activity was lower on day 60 PI in the blood of the infected mice (Fig. 3).

DISCUSSION

The present study reproduced *B. ovis* infection in mice, an experimental model capable of mimicking natural infection [21]. Because it is a facultative intracellular pathogen, *B. ovis* is able to persist in host cells and spread to organs such as the spleen, liver and other tissues [22]. Nevertheless, the inflammatory response has been described as moderate. In this study, we investigated the activity of cholinergic enzymes in the blood and brain of *B. ovis*-infected mice, because the cholinergic system has a role in the inflammatory response [23].

In this study, AChE activity in blood increased following *B. ovis* infection, as described in infections caused by *Leptospira interrogans* [24] and by protozoa such as *Trypanosoma cruzi* [25]. The mice infected by *B. ovis* developed a neutrophil-mediated inflammatory responses, associated with splenomegaly and tissue inflammatory responses, as previously described [12]. The increase in AChE activity observed in the present study generated a rapid degradation of ACh, a molecule with anti-inflammatory action since it binds to nicotinic receptors on leukocyte surfaces [23]. In this way, the molecule inhibits the proliferation of cytokines, serotonin, histamine, nitric oxide, lysosomal enzymes,

prostaglandins and leukotrienes that are mediators of inflammatory processes [23]. Therefore, because greater hydrolysis of ACh occurred, decreasing levels of this anti-inflammatory molecule [26], exacerbation of the inflammatory response may have occurred. An increase in serum BChE activity was also observed in the present study. This increase was probably due to late pro-inflammatory responses in order to reduce the concentration of ACh, as it exhibits anti-inflammatory properties as previously described [27]. Systemic infection by *B. ovis*, instead of causing hepatic tissue lesions, did not appear to affect the production of BChE in the liver, since this organ is involved mainly in BChE synthesis [28]. AChE and BChE both catalyze the hydrolysis of acetylcholine (ACh), the fundamental process in regulating the cholinergic system [29, 30]. Therefore, cholinesterases in the experimental infection by *B. ovis* have a pro-inflammatory role, as both contribute to reduction in the anti-inflammatory ACh molecule in the extracellular space.

With increased AChE activity in the brain, the hydrolysis of ACh in synaptic clefts is probably increased. Consequently, the reduction of this neurotransmitter and neuromodulator in the CNS [31, 32], due to increased degradation of ACh by AChE can lead to cerebral dysfunctions affecting behavior, memory, locomotion, balance and orientation, all of which are neurological disorders already described in patients with brucellosis [33]. In the literature, the increased AChE activity excessively hydrolyzes ACh in cholinergic synapses and/or neuromuscular junction, possibly leading to cognitive disorders, behavioral and functional related to a hypocholinergic state, as occurs for example in Alzheimer's disease [34], Parkinson's disease [35] and multiple sclerosis [36]. Therefore, a similar mechanism may occur in brucellosis, as observed in this study.

According to the literature, excess production of reactive substances such as ROS requires a response of endogenous antioxidants, including superoxide dismutase and catalase [37]. However, these enzymes cannot always be activated, and many of their activities may be

reduced, including CAT in the present study, resulting in a situation of oxidative stress. If the antioxidant response is not efficient and ROS levels remain high, tissue damage may occur due to damage to phospholipids, carbohydrates, amino acids, DNA or even cell death due to necrosis or apoptosis [38], thus contributing to clinical disease. The oxidative and antioxidant imbalance observed in experimental brucellosis in the present study may contribute to the pathogenesis of the disease, i.e., reproductive problems caused by the induction of genital lesions, epididymitis and low semen quality [39] in addition to subfertility and infertility in males [3]. In this context, the generation of low levels of ROS is necessary to regulate the activity of genes and proteins vital for sperm cell differentiation and function [40] and for the process of sperm capacitation [41], however, in high concentrations they can cause serious damage to both the cell membranes and sperm DNA. The sperm cell membrane is highly susceptible to oxidative stress because it contains a high concentration of unsaturated fatty acids that undergo a lipid peroxidation process, leading to loss of integrity and stability of the membrane lining the sperm cell, causing a dramatic decrease in motility and loss of sperm function. It also causes defects in the integrity of spermatic chromatin and the mitochondrial genome. As already mentioned, these alterations can be aggravated if there is a depletion of the antioxidant system, because thus more circulating free radicals can cross the nuclear or cellular membrane and induce damage through enzymatic reactions [42], reinforced in this study by the reduction of CAT activity. This situation of oxidative stress is probably related to the changes in the activity of cholinesterases in this study, as already described in the literature [43].

CONCLUSIONS

Cholinesterases may have proinflammatory actions during *B. ovis* infection, reducing concentrations of extracellular ACh, a potent anti-inflammatory mediator. The

reduction of ACh in the brain may also interfere with nerve synapses, and may be related to neurological disorders already described in patients with brucellosis. In addition, ovine brucellosis is capable of generating a picture of oxidative stress that may contribute to cellular and tissue damage, and consequently to clinical disease.

Conflicts of interest: The authors declare that there are no conflicts of interest.

CEUA: This experiment was approved by the Animal Welfare Committee of the State University of Santa Catarina (UDESC) under protocol number 4438310517.

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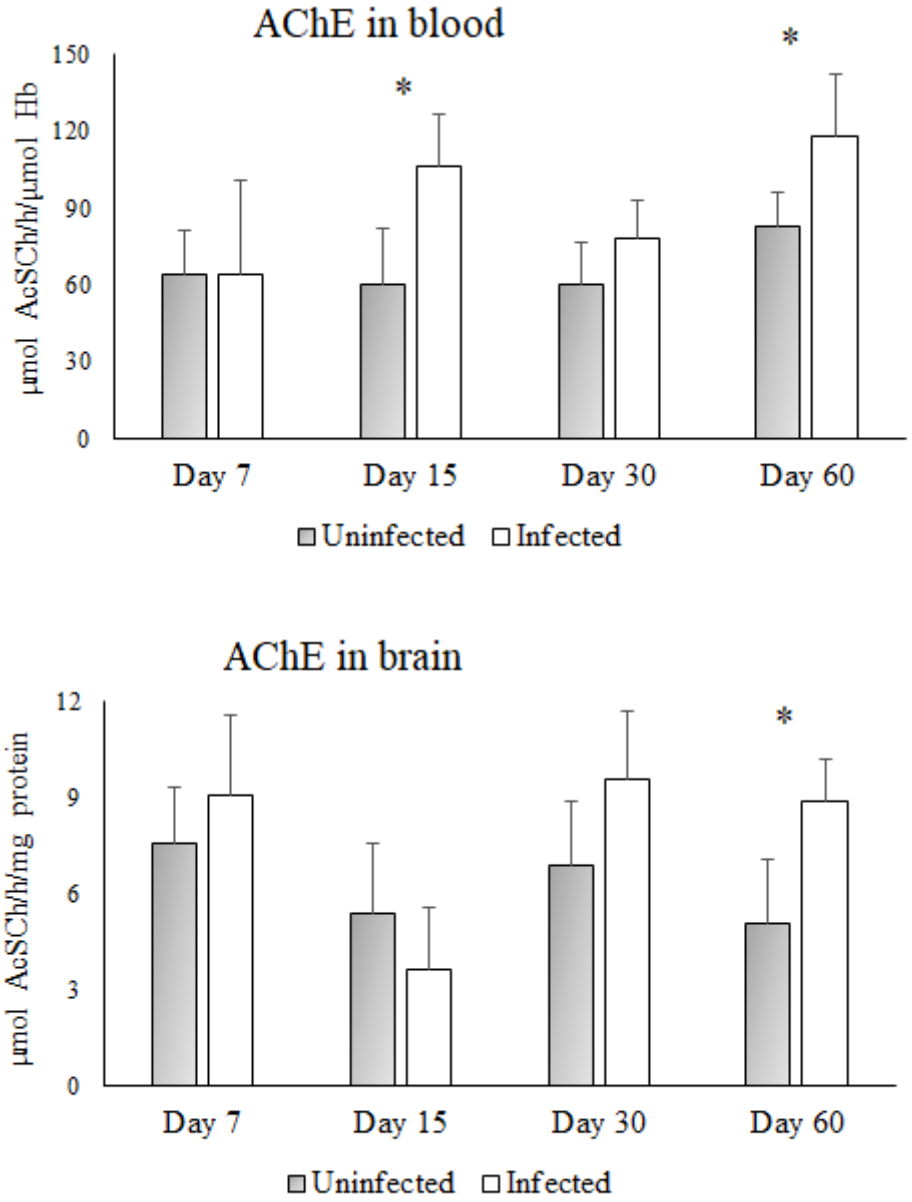

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Figure 1: Activity of the enzyme acetylcholinesterase (AChE) in whole blood and brain. Analysis performed on mice experimentally infected with *B. ovis* (n = 12) on days 7, 15, 30 and 60 post-infection (*P <0.05). Results are expressed as the mean ± standard deviation.

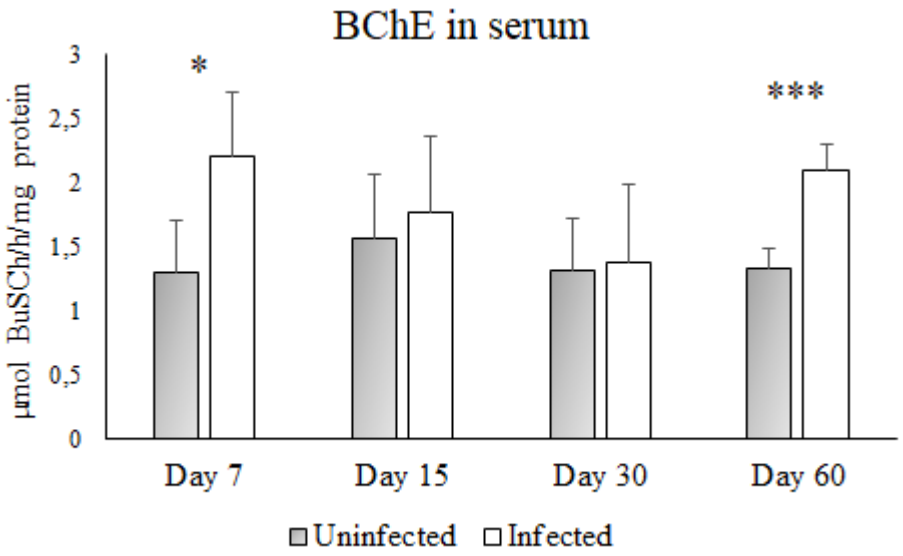

Figure 2: Enzymatic activity of butyrylcholinesterase (BChE) in serum. Analysis performed on mice experimentally infected with *B. ovis* (n = 12) on days 7, 15, 30 and 60 post-infection (*P <0.05 ***P). Results are expressed as the mean ± standard deviation.

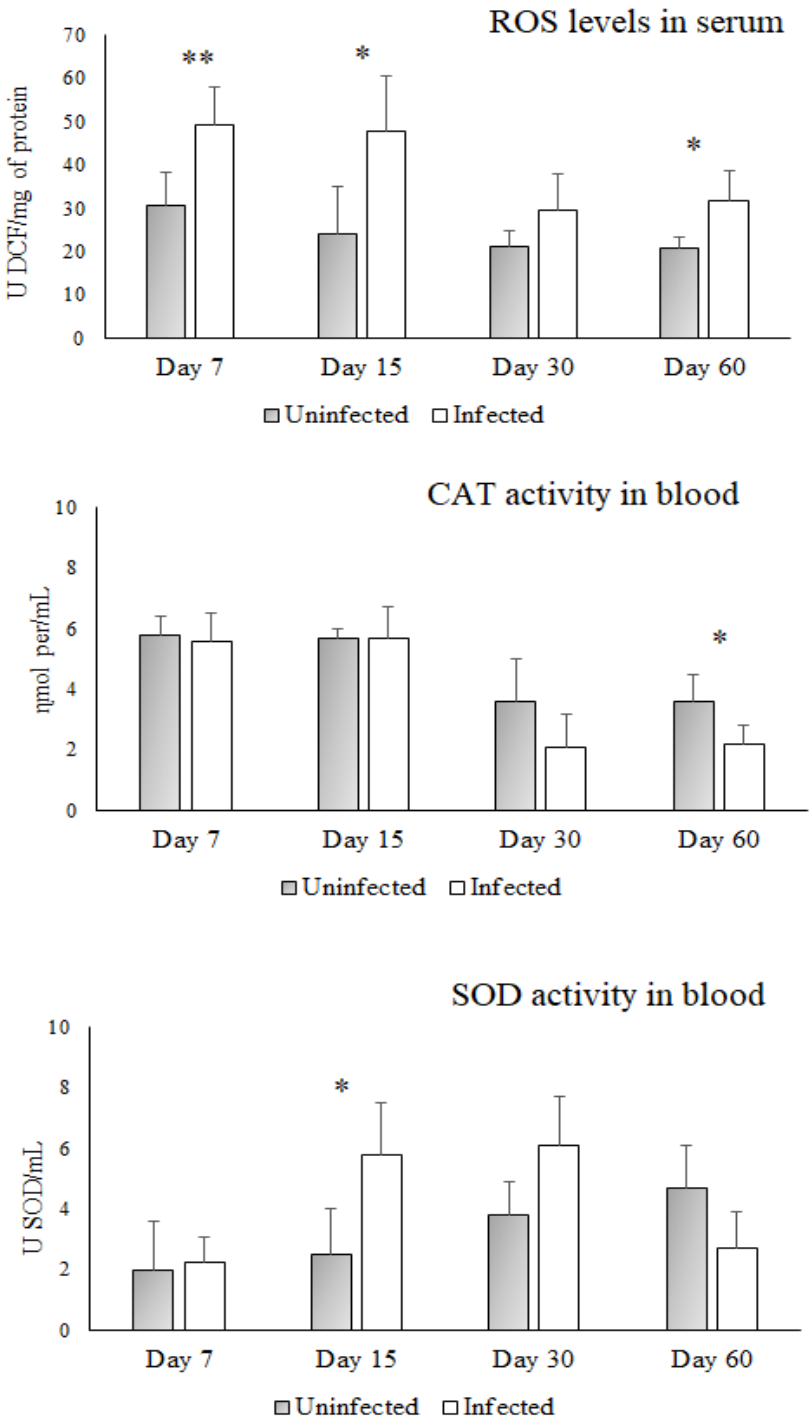

Figure 3: Levels of serum reactive substances (a: ROS), Catalase activity (b: CAT) and Superoxide dismutase (c: SOD) in whole blood. Analysis performed on mice experimentally infected with *B. ovis* (n = 12) on days 7, 15, 30 and 60 post-infection (**P* <0.05). Results are expressed as the mean ± standard deviation.

2.5 – MANUSCRITO II

Cerebral tissue of mice infected with *Brucella ovis* shows altered ectonucleotidase activity and increased levels of oxidative stress indicators

Géssica Perin[1], Anielen D. Silva[2], Nathieli B. Bottari[2], Letícia T. Druzian[2], Charles E. Assmann[2], Teane M.A. Gomes[3], Maria R.C. Schetinger[2], Vera M. Mosch[2], Aleksandro S. Da Silva[2]

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Abstract

Brucella ovis is a major cause of reproductive failure in sheep. It is associated with epididymitis and infertility in rams. However, this bacterium also causes neurological abnormalities that remain poorly understood. The purinergic system is thought to fine-tune immune cell functions, including cell-to-cell interactions, surface antigen shedding, intracellular pathogen removal and generation of reactive oxygen species (ROS). Therefore, the objective of this study was to investigate the role of ectonucleotidases during infection by *B. ovis* in the brains of experimentally infected mice. Forty-eight animals were divided into two groups: control (n = 24) and infected (n = 24). The cerebral cortexes were collected from the animals on days 7, 15, 30 and 60 post-infection (PI). Brain damage was assessed by levels of TBARS (substances reactive to thiobarbituric acid) and ROS (reactive oxygen species) in the brain. The activity of NTPDase (using ATP and ADP as substrate) and 5'-nucleotidase (using AMP as substrate) were evaluated in brain in addition to histopathological analysis. The data demonstrated an increase in lipid peroxidation and reactive species in the brains of infected mice compared to control animals, indicating cerebral oxidative damage. In addition, *B. ovis* infection increased the activity of NTPDase and 5'-nucleotidase at day 60 PI. The results demonstrated that with the chronicity of infection by *B. ovis*, there is modulation of ectonucleotidases, oxidative damage and inflammation in the brain.

Keywords: ATP; brucellosis; NTPDase; 5'-nucleotidase; oxidative stress.

1. Introduction

Brucellosis is one of the most significant causes of economic losses in cattle farming main (Jardim et al., 2006). In ewes, the ovine brucellosis can be caused by the Gram-negative coccobacillus *Brucella ovis,* as well other species zoonotic as *B. melitensis* and *B. abortus*. The clinical manifestations of *Brucella* sp. infections are most apparent in reproductive organs and reticuloendothelial tissue, leading to infertility, and associated with abortion in mammals (Ocholi et al., 2005). Hepatosplenomegaly have been described in ovine brucellosis (Silva et al. 2011; Perin et al. 2018), therefore, there is an extravascular inflammatory response that occurs in response to *B. ovis* infection. During the inflammatory processes, there is mobilization of immune cells to the central nervous system (CNS) in order to produce and release antibodies and cytokines (Kraal and Haan 2016), in addition to exacerbated inflammatory responses. There are no studies linking ovine brucellosis to neurological changes; however, cases of neurological manifestations and brain lesions in patients with brucellosis have been described (Türe et al. 2010; Dreshaj et al. 2016). In many cases of human brucellosis, neurological findings include agitation, behavioral disorders, disorientation and stroke (Dreshaj et al. 2016), however, the mechanisms involved have not been described. In sheep infected with *B. ovis* there is no record of neurological signs or behavioral changes, which may be due to the lack of studies related to the subject; or bacterium *B. ovis* is not pathogenic (smooth-walled bacteria), other than the pathogenic (rough wall) that caused neurological disease in humans.

Changes in purinergic signaling have appeared in various pathologies associated with inflammation. Purinergic signaling is an important modulating pathway for a variety of physiological processes involved in many neuronal and non-neuronal mechanisms and in short- and long-term events, including immune responses, inflammation, endothelium-mediated vasodilation, proliferation and cell death (Burnstock 2006). A recent study showed

reduction in the expression of NTPDase (CD39) in RAW cells and intraperitoneal macrophages infected with *Brucella abortus* (Melo 2017). According to the literature, numerous physiological functions are involved by the interaction of extracellular purines with specific cellular receptors in cells and tissues (Ralevic and Burnstock 1998).

NTPDases and 5'-nucleotidase are enzymes involved in purinergic signaling responsible for controlling purine levels, playing an important role in physiological processes, as well as in inflammatory diseases (Schetinger et al. 2007). Among the functions of NTPDase are inhibition of platelet aggregation (Gayle et al. 1998), vascular homeostasis (Atkinson et al. 2006), modulation of inflammation and immune response, because it regulates extracellular concentrations of ATP (Mizumoto et al. 2002), a pro-inflammatory molecule (Bours et al. 2006). E-NTPDase plays an important role in controlling lymphocyte function, including antigen recognition and activation of cytotoxic T cell effector functions (Filippini et al. 1990), as well as the ability to generate signals that amplify cell-cell interactions (Kaczmarek et al. 1996). The enzyme E-5´-nucleotidase also exerts non-enzymatic functions, including induction of intracellular signaling and mediation of cell-cell adhesion and cell-matrix and migration (Vogel et al. 1991).

Considering the purinergic system enzymes as being closely involved with the modulation of the immune system, participating in the regulation of both pro- and anti-inflammatory events, the objective of this study was to investigate the role of ectonucleotidases and oxidative damage in the brains of mice experimentally infected with *B. ovis*.

2. Materials and methods

2.1. Inoculum

A virulent *B. ovis* strain (ATCC 25840), also known as NCTC10512 or 63/290, was used in this present study. The inoculum preparation was performed following the methodology described by Perin et al. (2018).

2.2 Animals and samples

Forty-eight frozen mouse brain samples (*Mus musculus,* Swiss lineage; 60 days old; 30 ± 0.5 g) from a previous study (Perin et al. 2018) were used in the present study. The animals were maintained in cages under controlled temperature and humidity (25 ºC, 70 %), fed with a commercial feed and water *ad libitum.* The animals were divided into two groups of twenty-four animals each: uninfected mice (the control group) and *B. ovis* experimentally infected mice (100 µL containing 1.3 x 10^7 UFC/mL via intraperitoneal). The control group received 100 µL of phosphate buffer solution (PBS) intraperitoneally. All animals were observed daily.

Six animals from each group were euthanized on days 7, 15, 30 and 60 post-infection (PI) after anesthesia with isoflurane in an anesthetic chamber. Then, the animals were humanely euthanized by cervical dislocation. The spleen was increased in all infected and positive animals by PCR for *B. ovis* as previously described by Perin et al. (2018). Cerebral tissue was collected for measurement of enzymatic and histopathological analyses.

2.3. Sample preparation

Brains were weighed and placed in test tubes. The tissue was homogenized in Tris–HCl 10 mmol, pH 7.2 with 160 mmol sucrose (1:10 w/v). The corrected protein levels (0.8-

1.0 mg/mL), were evaluated by the Coomassie Brilliant Blue binding assay. All procedures described above were performed at 4ºC.

2.4 Lipid peroxidation

Lipid peroxidation was determined by concentration of malondialdehyde (MDA) (Ohkawa et al. 1978). The absorbance was measured at 532 nm. The amount TBARS (thiobarbituric acid levels) was expressed as µmol MDA/mg protein.

2.5 Reactive oxygen species (ROS) levels

Reactive oxygen species were measured by 2'-7'-dichlorofluresceina (DCFH) in brain as previously reported (Halliwell and Gutteridge 2007). The samples were incubated with DCFH 1 mM at 27 ºC over 30 minutes. The reaction was read on a fluorimeter (emission 525 nm and excitation 488 nm), and the results were expressed as U DCFH/mg of protein.

2.6 NTPDase and 5' nucleotidase activities

ATP and ADP hydrolysis were evaluated by E-ATPase total enzyme in brain homogenates as described in a modified method (Schetinger et al. 2000). The reactions were carried out in a medium containing KCl (5 mmol/L), $CaCl_2$ (1.5 mmol/L), EDTA (0.1 mmol/L), glucose (10 mmol/L), sucrose (225 mmol/L) and Tris–HCl buffer (45 mmol/L, pH 8.0), in a final volume of 200 µL. The hydrolysis of AMP was evaluated by a method described previously (Heymann et al. 1984; Chan et al. 1986) in a reaction medium containing $MgSO_4$ (10 mmol/L) and Tris–HCl buffer (100 mmol/L, pH 7.5). The enzyme preparations (0.8–1.2 mg of protein) were pre-incubated at 37ºC for 10 min using ATP or ADP as a substrate in a final concentration of 1.0 mmol/L or AMP at a final concentration of

2.0 mmol/L. All samples were run in triplicate. The data were reported as nmol Pi released/min/mg of protein.

2.7 Histopathology

One sample of cerebral cortex at each time point was fixed in formalin solution (10%) for two days and then transferred to 70% alcohol solution. Sagittal sections were embedded in paraffin and stained with Hematoxylin and Eosin.

2.8 Statistical analysis

The data were first subjected to the normality test and transformed to logarithms. The results were expressed as mean and standard deviation. Data were analyzed by Student test (t-test) using GraphPad Prism (version 6). The results were considered statistically significant when $p<0.05$ between groups.

3. Results

3.1 Lipid peroxidation and reactive oxygen species in brain

During the course of infection, *B. ovis* caused greater lipid peroxidation in the brains of infected animals (Fig. 1A) than in the control group ($p<0.05$) at day 60 PI. No significant results were observed at 7, 15 or day 30 PI. Similarly, there was significantly more reactive oxygen species at day 60 PI in brains of infected animals (Fig. 1B) than in the control group.

3.2 Ectonucleotidases in brain

NTPDase and 5´nucleotidase activities are shown in Figure 2. NTPDase activity (using ATP and AMP as substrate) was lower at days 7 and 15 PI in infected animals than in

control ($p<0.05$). However, during the course of infection there was an increase in NTPDase activity at day 60 PI in the infected group.

The infected animals showed a decrease of 5´-nucleotidase (AMP as substrate) activity at days 7 and 30 PI. On the other hand, 5´-nucleotidase activity was greater on day 60 PI in the experimental group than in the control. The results suggest that nucleotide hydrolysis was low in the acute phase (up to day 30 PI) due to the decrease of NTPDase and 5´-nucleotidase activities. After day 60 PI, there was a reversal in enzyme activities, probably with concomitant increase of extracellular nucleotides.

3.3 Histopathology

No histopathological lesions were observed in the control group nor the infected group at days 7, 15, and 30 PI. However, multifocal areas with moderate microgliosis and inflammatory infiltrates in the cerebral cortex were observed at day 60 PI of infected animal (Fig. 3).

4. Discussion

The capacity of *B. ovis* to successfully survive and replicate in host cells is critical to their virulence. *Brucella* spp. employ several strategies to establish and maintain persistent intracellular in host cells. The bacterium is able to avoid a full-blown inflammatory response during the initial stages of infection in order to remain in the CNS. In this study, we evaluated the role of ectonucleotidases and oxidative damage in cortex of mice experimentally infected with *B. ovis*. However, neurological clinical signs were not observed in either the control animals or in mice infected by *B. ovis* during the experiment.

Our results show that the *B. ovis* infection induced oxidative damage with an increase of reactive species and lipid peroxidation. During the course of disease (i.e., at 60 days PI)

there were increases in ROS and TBARS levels in the brains of infected mice. Elevation of these factors in mice infected with *B. ovis* indicates an increase in extracellular free radicals and lipid peroxidation. This process damages cell membranes, reduces membrane fluidity and increases extracellular permeability (Gill 1931). It is known that increased production of ROS leads to cell death and apoptosis when there is no elevation in antioxidant defense enzymes that are responsible for neutralizing reactive species (Cornelius et al. 2013; Rodriguez-Rodriguez et al. 2014). *Brucella* spp. strains generate ROS such as O_2^- and H_2O_2 endogenously as a consequence of their aerobic respiratory-type metabolism (Jiang et al. 1993; Hop et al. 2018). Exogenous production of these ROS has also been shown to be important for the brucellacidal activity of macrophages (Jiang et al. 1993; Hop et al. 2018). In tissues damaged by infections due to a series of inflammatory and oxidative processes, the production of free radicals is high. This was observed in our study with chronification of the disease contributing to oxidative damage and inflammatory responses in the brains of infected animals.

E-NTPDase and 5´-nucleotidase enzymes are essential to the regulation of inflammatory processes mediated by extracellular ATP and nucleotides. We found decreased NTPDase and 5'-nucleotidase enzymes activities in infected animals in the acute phase of infection (days 7 and 15 PI), followed by increased activity as the infection progressed (day 60 PI). The decreased on NTPDase for ATP during the acute phase of disease can be considered a pro-inflammatory effect due the reduction on ATP hydrolyses in the extracellular medium, in accordance to observed by da Silva et al. (2017) in serum of cows naturally infected by *Dictyocaulus viviparus.* According to these authors, the downregulation on NTPDase activity by ATP exerts a pro-inflammatory profile due to the augmentation of ATP levels, that induces the release of pro-inflammatory cytokines, and consequently contributes to disease pathophysiology. The increase in NTPDase activity is a direct

reflection of the increase in ATP hydrolysis and, consequently, the reduce in the concentrations of this molecule in the extracellular space. The increased E-NTPDase and 5'-nucleotidase activities imply their involvement in an anti-inflammatory response and modulatory response through augmentation on hydrolyzes of the excessive ATP content in the extracellular medium, preventing excessive tissue damage caused by excessive ATP levels during *B. ovis*, in accordance to observed by Souza et al. (2018) in brain of silver catfish experimentally infected by *Streptococcus agalactiae.* According to these authors, the augmentation on NTPDase activity elicited a reduction on ATP content, and consequently minor interaction of ATP with P2X7 purinereceptor, which reduced the release of pro-inflammatory mediators. ATP is an important pro-inflammatory agent involved in vasodilation and platelet activation and it stimulates the production of reactive species, thus directly stimulating immune cells (Bours et al. 2006) in order to remove invading pathogens. The roles of ADP and AMP during inflammation remain unknown. In our experimental study, ADP and AMP hydrolysis was significantly increased in the infected animals at day 60 PI, probably leading to decreases in extracellular concentrations. Augmentation on ADP and AMP hydrolyzes can be considered an attempt to produce more adenosine, a molecule with anti-inflammatory and immunosuppressive properties, as evidenced by Souza et al. (2018) in brain of fish experimentally infected by *S. agalactiae.* According to these authors, increase in AMP hydrolysis by 5'-nucleotidase may be considered as an anti-inflammatory effect in order to augments the production of adenosine, as also observed by Doleski et al. (2016) in serum and liver of rats experimentally infected by *Fasciola hepatica.*

Histopathological changes were consistent with multifocal areas of moderate microgliosis at day 60 PI. This set of alterations may be associated with the pathogenesis of the neurological manifestations described in cases of brucellosis, which can be explained by the fact that, when CNS homeostasis is altered by pathogens or by injury, as in brucellosis,

microglial cells (macrophages in the CNS) respond rapidly with changes in their morphology, expression of surface proteins, gene expression and proliferation leading to microgliosis (Hong et al. 2016). Together, the data here demonstrated that a with the chronicity of infection (up to day 30 PI) by *B. ovis* causes oxidative damage in the brain of infected animals, as well as alter the activity of purinergic enzymes possibly modulating extracellular ATP concentrations and contributing to CNS inflammation. The elucidation of this mechanism points to new therapeutic targets for the treatment and prevention of *B. ovis* infection.

5. Conclusion

The results led us to conclude that *B. ovis* infection causes changes in activity of ectonucleotidases, that may be involved in inflammatory and neurological mechanisms in the pathogenesis of *B. ovi*s, with a proinflammatory response in the acute and anti-inflammatory responses with the chronicity of infection, when it coincides with the appearance of microscopic cell lesions, associated with lipid peroxidation and increased free radicals in the brain.

Ethics Committee: These experiments were approved by the Animal Welfare Committee of the State University of Santa Catarina (UDESC) under protocol number 4438310517.

Conflict of interest

The authors have declared no conflict of interest.

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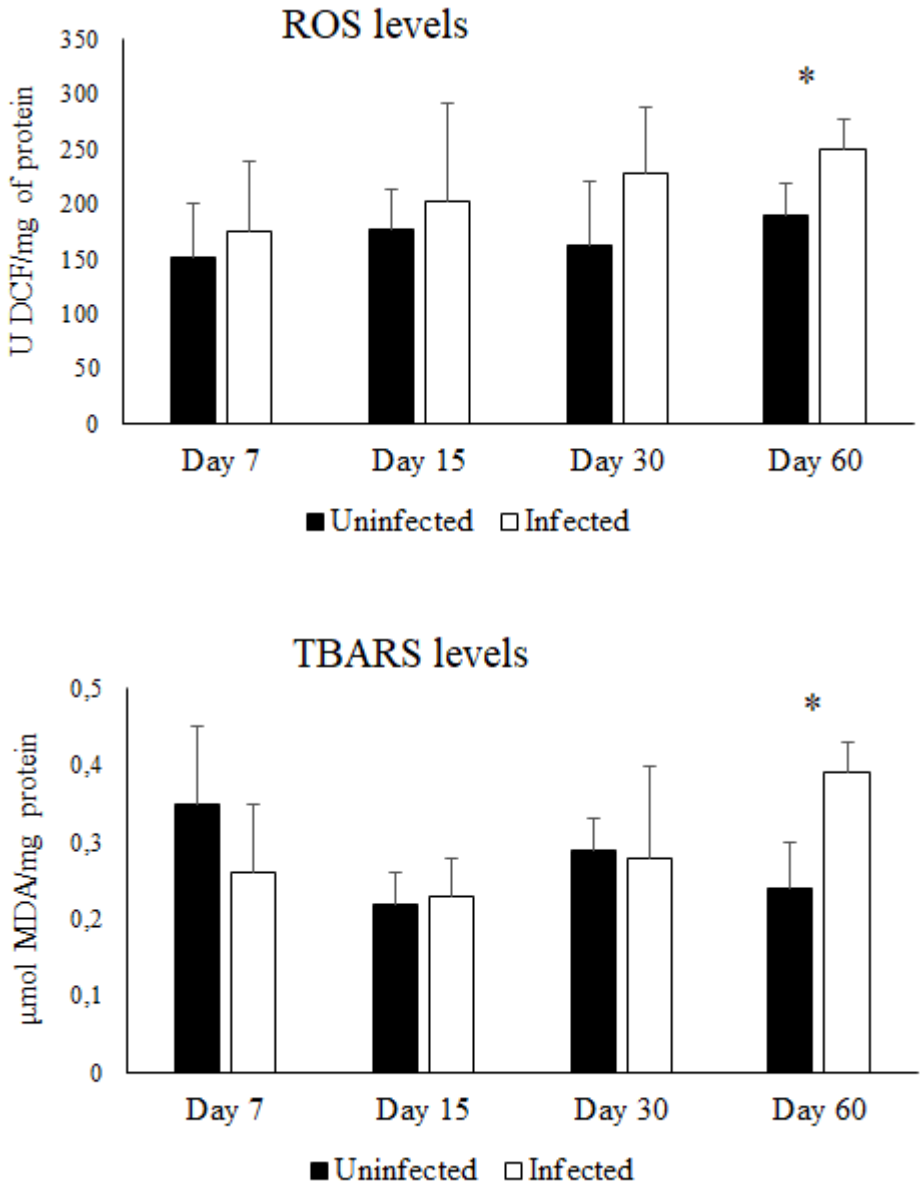

Figure 1- *B. ovis* induced oxidative damage during the course of infection. A. Reactive species (RS). **B.** Substances reactive to thiobarbituric acid (TBARS) of control and infected in mouse brains. Sample collection on days 7, 15, 30 and 60 post infection. The results are expressed as mean± standard deviation, using the t-test. The results are considered statistical significant at *p<0.05.

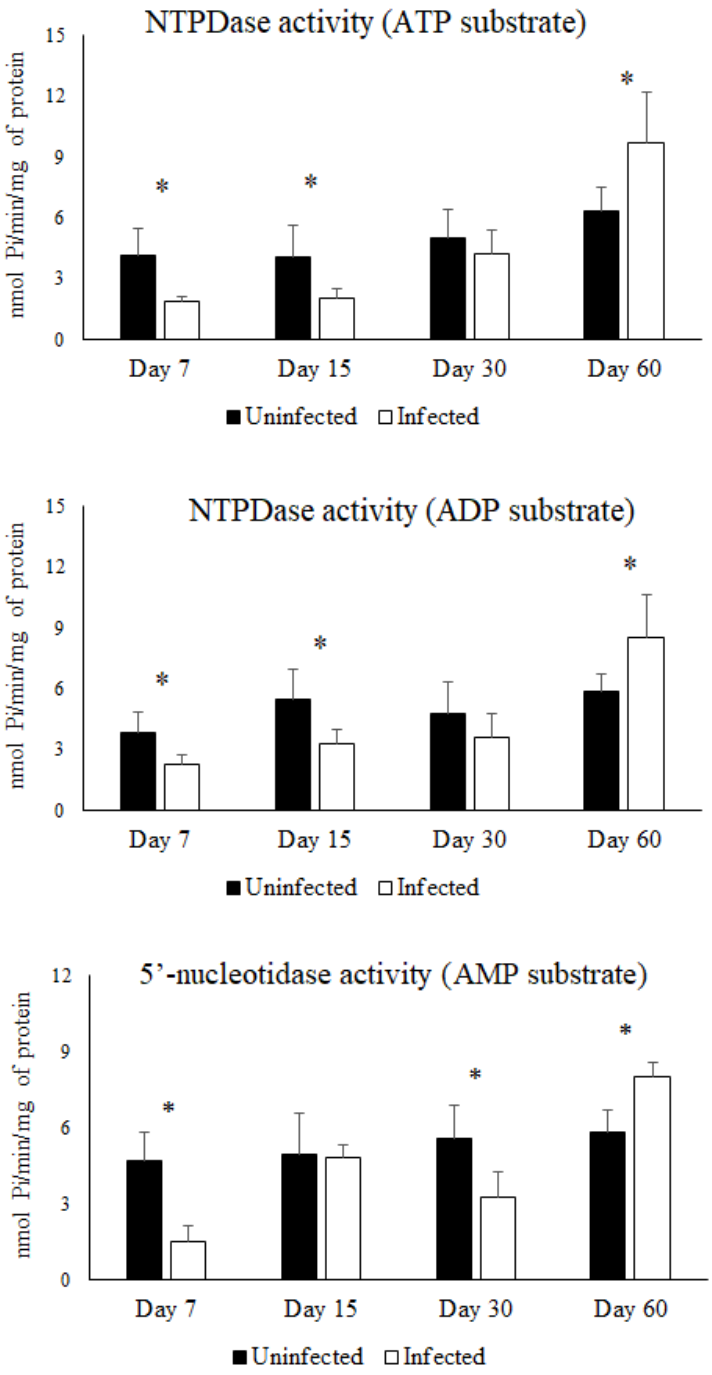

Figure 2- *B. ovis* modified activity of ectonucleotidases in brain of infected mice. NTPDase and 5´-nucleotidase activities of infected and control groups at days 7, 15, 30 and 60 post infection. The results are expressed as mean± standard deviation, using the t-test. The results are statistical significant at *$p<0.05$.

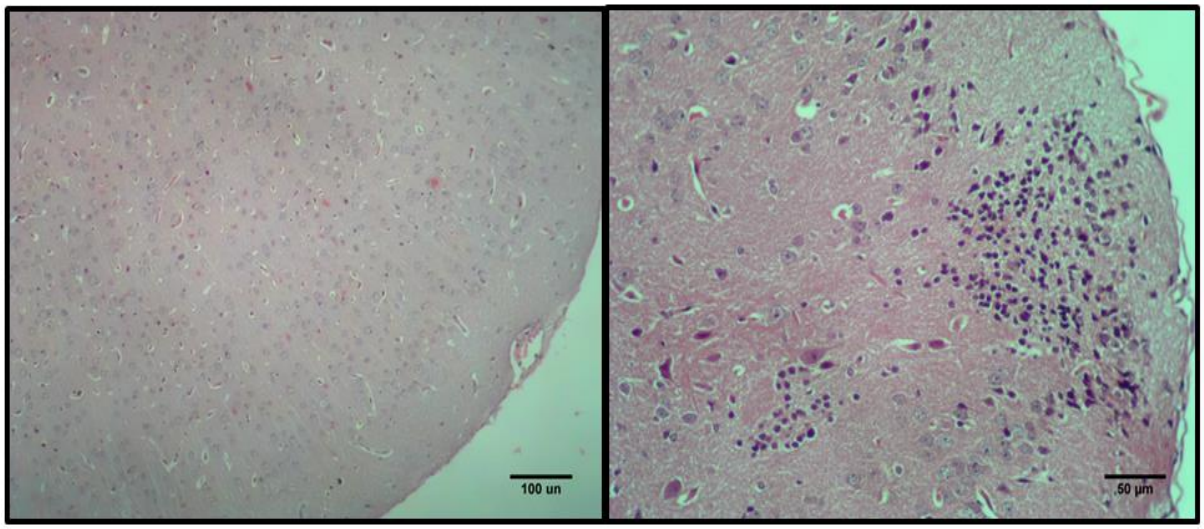

Figure 3 - Encephalic lesions in mice experimentally infected with *Brucella ovis*. (A) Normal cortex, control group. HE, 100x. (B) Cerebral cortex with multifocal areas of moderate microgliosis in one mice, 60 days of infection. HE, 200x.

2.6 – MANUSCRITO III

Impairment of cardiac creatine kinase and pyruvate kinase activities in *Brucella ovis* infection

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ABSTRACT

The aim of this study was to evaluate whether *Brucella ovis* infection altered the activities of cardiac enzymes belonging to the phosphotransfer network and to evaluate the implications of this infection on disease pathogenesis in experimental conditions. We used hearts from 48 mice in two groups (n = 24 each): uninfected (control group) and infected by *B. ovis*, collected on days 7, 15, 30 and 60 post-infection (PI). Cardiac cytosolic creatine kinase (CK) activity was higher in infected animals than in the control group on days 30 and 60 PI, while cardiac mitochondrial CK activity was lower at the same moments of infection. Cardiac pyruvate kinase (PK) activity was lower in infected animals than in the control group on day 60 PI, while no significant difference was observed between groups regarding cardiac adenylate kinase (AK) activity. Cardiac thiobarbituric acid reactive substances (TBARS) were higher in infected animals than in the control group on days 30 and 60 PI, while non-protein thiol (NPSH) levels were lower at the same moments of infection. Finally, cardiac SOD activity was higher in infected animals than in the control group on day 60 PI. Based on this evidence, inhibition on mitochondrial CK activity caused an impairment of cardiac energy homeostasis, possibly through depletion of ATP production, although cytosolic cardiac CK activity acted in an attempt to restore the mitochondrial ATP levels through a feedback mechanism. In summary, *B. ovis* infection caused a severe energetic imbalance in infected mice, possibly contributing to disease pathogenesis.

Keywords: energy metabolism; heart; oxidative stress; disease pathophysiology; ATP.

1. Introduction

Ovine brucellosis by *Brucella ovis* is a chronic infectious disease caused by Gram-negative bacteria that affects the reproductive tract in affected animals [1,2]. This bacterium preferentially colonizes epididymal tissue, but it can circulate throughout the body, and can colonize cardiac tissue [3]. Cardiac complications have been reported in humans affected by brucellosis, including systolic murmur, mild cardiomegaly and increased levels of cardiac lesion biomarkers such as creatine phosphokinase, lactate dehydrogenase and troponin [4,5]. Nevertheless, the mechanisms involved in the pathogenesis of cardiac lesions remains unknown, though they are putatively linked to cardiac energetic metabolism.

Cardiac muscle is highly-dependent on mitochondria to provide energy and blood to all organs of the body [6]; mitochondria produce 90% of cellular energy by generating adenosine triphosphate (ATP) [7]. ATP is a direct source of energy suitable for cardiac physiological functions, and the enzymes of the phosphotransfer network, particularly creatine kinase, have an essential role in the bioenergetic homeostasis between ATP synthesis and ATP consumption [8].

The phosphotransfer network is fundamental to cardiac bioenergetic homeostasis, with a significant role in heart energetics by catalysis of nucleotide exchange, facilitating intracellular energy communication in order to maintain a balance between cellular ATP consumption and production [9,10]. CK catalyzes the reversible transfer of a phosphoryl group from ATP to adenosine diphosphate (ADP) and creatine to produce phosphocreatine (PCr), considered a central controller of energy homeostasis in order to prevent a reduction on ATP levels [11]. PK is the key enzyme in the glycolytic pathway, catalyzing the irreversible transfer of phosphoenolpyruvate (PEP) to ADP to form pyruvate and ATP, the principal provider of energy for cellular and tissue functioning [12]. Finally, AK catalyzes the reversible transfer of the γ-phosphate group to adenosine monophosphate (AMP), releasing

two molecules of ADP, playing a significant role for processing and delivering metabolic signals in sites of ATP synthesis and utilization [13]. In this sense, a recent study [14] demonstrated that impairment of enzymes in the phosphotransfer network was involved in the pathogenesis of cardiac lesions in rats experimentally infected by *Trypanosoma evansi*. Therefore, our hypothesis as that alterations of the cardiac phosphotransfer network may be a pathway of *B. ovis*-induced cardiac tissue damage.

Thus, the aim of this study was to evaluate whether *B. ovis* infection alters the activities of cardiac enzymes of the phosphotransfer network and the implications of this infection on disease pathogenesis in experimental conditions.

2. Material and methods

2.1 Animals, experimental study and sample collection

We used frozen heart fragments of 24 mice experimentally infected via intraperitoneal injection with 100 µL of *B. ovis* suspension containing 1.3 x 10^7 CFU/mL [15], as well as hearts of 24 healthy mice as a control group. The animals were euthanized with isoflurane in an anesthetic chamber at days 7, 15, 30 and 60 PI. At these time points, we collected hearts for histopathology and biochemical analyses.

2.2 Tissue homogenization

The fragments of hearts were washed in SET buffer (0.32 M sucrose, 1 mM EGTA, 10 mM Tris-HCl, pH 7.4) and homogenized (1:10 w/v) in SET buffer with a Potter-Elvehjem glass homogenizer. The homogenates were centrifuged at 800 × *g* for 10 min at 4 °C. Part of the supernatants were used to evaluate AK activity; the pellet was discarded and the supernatant was once again centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatants containing cytosol were collected for determination of PK and cytosolic CK activity. The

pellet, containing mitochondria, was washed twice with SET buffer, then resuspended in 100 mM Trizma and 15 mM $MgSO_4$ buffer (pH 7.5) to evaluate mitochondrial CK activity. The supernatants were stored for no more than 1 week at −80 °C.

2.3 CK, AK and PK activities

CK activity was assayed in the reaction mixture containing the following final concentrations: 65 mM Tris-HCl buffer, pH 7.5, 7 mM PCr, 9 mM $MgSO_4$ and 1 μg of protein in a final volume of 150 μL. After 10 min of pre-incubation at 37 °C, the reaction was started by the addition of 0.3 μmol of ADP, and was stopped after 10 min by the addition of 1 μmol of ρ-hydroxymercuribenzoic acid. The creatine levels were estimated according to the colorimetric method [16]. The color was developed by the addition of 0.1 mL of 2% α-naphthol and 0.1 mL of 0.05% diacetyl in a final volume of 1 mL, and was read at 540 nm after 20 min. Results were expressed as nmol of creatine formed per min per mg protein.

AK activity was measured with a coupled enzymatic assay using hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PD), according to authors [17]. The reaction mixture contained 100 mM KCl, 20 mM HEPES, 20 mM glucose, 4 mM $MgCl_2$, 2 mM $NADP^+$, 1 mM EDTA, 4.5 U/mL of HK, 2 U/mL of G6PD and 20 μL of homogenate. The reaction was initiated by the addition of 2 mM ADP, and the reduction of $NADP^+$ was evaluated at 340 nm for 3 min in a spectrophotometer. ADP, $NADP^+$, G6PD and HK were dissolved in Milli-Q-purified water. The concentration of the reagents and the assay time (3 min) were chosen to assure linearity of the reaction. The results were expressed as pmol of ATP formed per min per mg protein.

PK activity was assayed essentially as described [18]. The incubation medium consisted of 0.1 M Tris/HCl buffer, pH 7.5, 10 mM $MgCl_2$, 0.16 mM NADH, 75 mM KCl, 5.0 mM ADP, 7 U L-lactate dehydrogenase, 0.1 % (v/v) Triton X-100 and 20 μL of the

mitochondria-free supernatant in a final volume of 500 µL. After 10 min of pre-incubation at 37 °C, the reaction was started with the addition of 1 mM PEP. All assays were performed in duplicate at 25 °C. Results were expressed as µmol of pyruvate formed per min per mg protein.

2.4 TBARS levels

As an index of lipid peroxidation, TBARS formation during an acid-heating reaction was determined as previously described [19]. Malondialdehyde (MDA) solution was used as a reference standard. TBARS levels were determined by the absorbance at 532 nm and were expressed as MDA equivalent (nmol MDA/mg protein).

2.5 NPSH levels

For measurement of NPSH levels, the hearts were homogenized (1:10 w/v) in a glass Potter tube with Tris-HCl buffer (10 mM, pH 7.4), and centrifuged at 2000 *x g* for 10 min. Cardiac NPSH levels were determined colorimetrically at 412 nm as previously described [20], and published in detail [21]. A cysteine solution was used as reference standard. NPSH were expressed as µmol SH/g tissue.

2.6 SOD activity

SOD activity was spectrophotometrically evaluated as described [22], recently published in detail [23]. The enzymatic activity was expressed as SOD units/mg of protein.

2.7 Histopathology

One cardiac sample by group were collected on days 7, 15, 30 and 60 PI and were preserved in 10% formaldehyde solution. The samples were placed in paraffin blocks

followed by staining with hematoxylin-eosin (HE), to be analyzed by light microscopy. The lesions were classified as absence, mild, moderate, severe and very severe.

2.8 Statistical analysis

The data were subjected to normality testing using the Shapiro-Wilk test, and did not present normal distribution. Therefore, the data were transformed to logarithmic form and the comparisons was performed using the *Student's t* test considering $P<0.05$. Results were expressed as average ± standard deviation.

3. Results

Cardiac cytosolic CK activity was higher in infected animals than in the control group on days 30 and 60 PI, while cardiac mitochondrial CK activity was lower at the same moments of infection. Moreover, cardiac PK activity was lower in infected animals than in the control group on day 60 PI, while no significant difference was observed between groups regarding cardiac AK activity (Figure 1).

No microscopic or macroscopic lesions were observed in either group during the entire experimental period (data not shown). Cardiac TBARS levels were higher in infected animals than in the control group on days 30 and 60 PI, while NPSH levels were lower on day 60 PI. Finally, cardiac SOD activity was higher in infected animals than in the control group on day 60 PI (Figure 2).

4. Discussion

The present study is novel, since it evaluated several alterations of the cardiac phosphotransfer network of mice experimentally infected with *B. ovis*. Our data clearly demonstrated an impairment in energetic homeostasis between sites of ATP utilization and

ATP consumption, possibly leading to a decrease in cardiac ATP availability and possibly involving pathological mechanisms related to cardiac alterations during brucellosis infection.

At 30 and 60 days, *B. ovis* infection elicited severe inhibition of cardiac mitochondrial CK activity, indicating an impairment in ATP production in a tissue with intermittently high and fluctuating energy requirements and in an organelle linked directly linked to energy production. This accords with observations [24] in the gills of silver catfish *Rhamdia quelen* experimentally infected by another Gram-negative bacterium, *Citrobacter freundii*. In addition, the inhibition of mitochondrial CK activity indicated an inability of PCr to form ATP in order to restore mitochondrial energetic homeostasis [25]. It is important emphasize that the existence of microcompartments of CK isoenzymes in sites of energy production (mitochondria) and energy consumption (cytosol) are considered key to maintenance of cellular energetic homeostasis [26], i.e., the existence of a reciprocal compensatory mechanism between compartments occurs in order to safeguard cellular energy economy. In this sense, we observed that cardiac cytosolic CK activity was stimulated by *B. ovis* infection, possibly a compensatory mechanism acting as a temporal and spatial energy buffer to maintain cellular energy homeostasis and to restore ATP levels, as observed [27] in silver catfish fed with a diet contaminated by aflatoxin B_1. Therefore, *B. ovis* infection inhibited mitochondrial CK activity, leading to an imbalance of energy homeostasis that was compensated for by stimulation of cytosolic CK activity.

A significant reduction of cardiac PK activity was observed in mice experimentally infected by *B. ovis* after 60 days of infection, provoking a decrease in the availability of cardiac ATP content and impairment of communication between sites of ATP generation and ATP utilization, in agreement with observations in gills of experimentally infected silver catfish with the Gram-negative bacterium *Pseudomonas aeruginosa* [28]. According to these authors, the inhibition on PK activity contributed directly to disease pathogenesis and

occurrence of branchial lesions, particularly destruction of primary and secondary lamellae. In summary, the inhibition of cardiac PK activity led to impairment of energy metabolism during *B. ovis* infection, contributing to disease pathophysiology linked to cardiac lesions.

A study [29] demonstrated that activity of enzymes belonging to the phosphotransfer network were highly susceptible to free radicals and oxidative damage. In this sense, we evaluated some parameters associated with oxidative stress in order to identify a possible pathway involved in the impairment of cardiac energetic metabolism. We observed an increase in cardiac lipid peroxidation in infected animals, as reported [30] in plasma of patients affected by brucellosis and in mice experimentally infected by *B. abortus* [31]. According to the authors [32], the augmentation of lipid peroxidation induced damage to cell membranes and to increases in extracellular fluidity, contributing to disease pathogenesis [33]. The damage to lipids may be related to the impairment of CK and PK activities observed in the present study. Studies [34] demonstrated that oxidative damage to proteins was directly related to the inhibition of CK activity during exposure to methylmercury, in agreement to the observations of the present study. In addition, it is important to emphasize that a thiol group is present in the catalytic site of CK and exerts a putative role in the protection against inactivation of free radicals, developing a key role for suitable enzymatic CK activity [35]. In this sense, the lower levels of the thiol group in cardiac tissue on days 30 and 60 of infection could contribute directly to the inhibition of cardiac mitochondrial CK activity observed at the same moment of infection. A study [36] demonstrated that oxidative stress caused inhibition of PK activity. This inhibition implicates a reduction in pyruvate content, impairing cell and tissue homeostasis [37]. Finally, cardiac SOD activity was stimulated in infected animals on day 60 PI, an effect that can be considered a defense mechanism against excessive production of free radicals [38], in agreement with observations in serum of dairy cows seropositive for *B. abortus* [39]. According to these authors, stimulation of SOD activity contributed to the

abolition of ROS production, possibly contributing to prevention or reduction of lesions provoked by disease.

Based on this evidence, the data demonstrated that *B. ovis* severely affected CK activity, an essential enzyme for maintenance cellular energy homeostasis in the heart. In addition, the inhibition of cardiac CK and PK activities appears to be mediated by the oxidation of lipids and thiol groups. In summary, the impairment of the cardiac phosphotransfer network may be a pathway involved in the disease pathogenesis of brucellosis.

Conflicts of interest

The authors declare that they have no conflicts of interests.

Ethics Committee

This experiment was approved by the Animal Welfare Committee of the State University of Santa Catarina (UDESC) under protocol number 4438310517.

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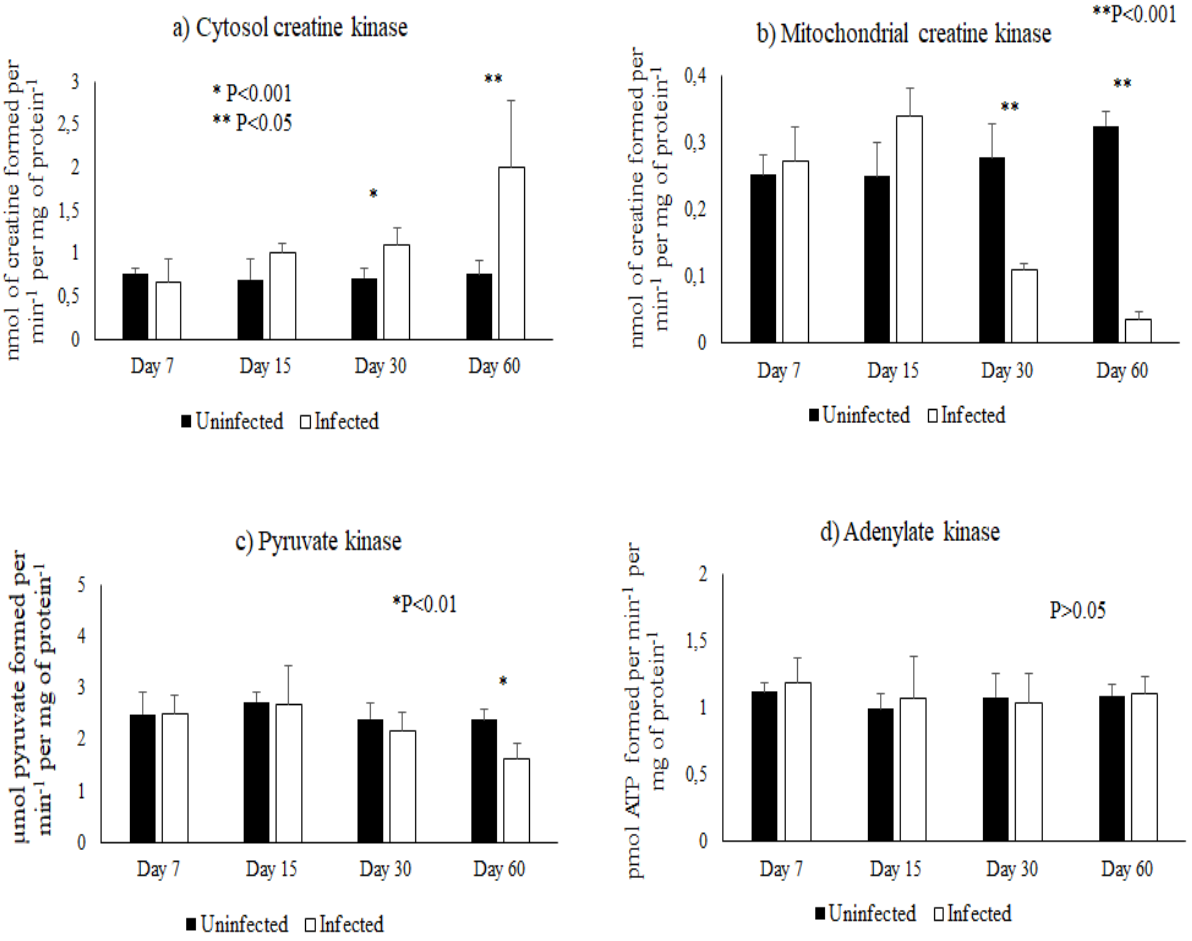

Figure 1: Cardiac cytosolic [A] and mitochondrial [B] creatine kinase (CK), pyruvate kinase (PK) and adenylate kinase (AK) activities of mice experimentally infected with *Brucella ovis* on days 7, 15, 30 and 60 post-infection (PI). **P*<0.01; ***P*<0.05.

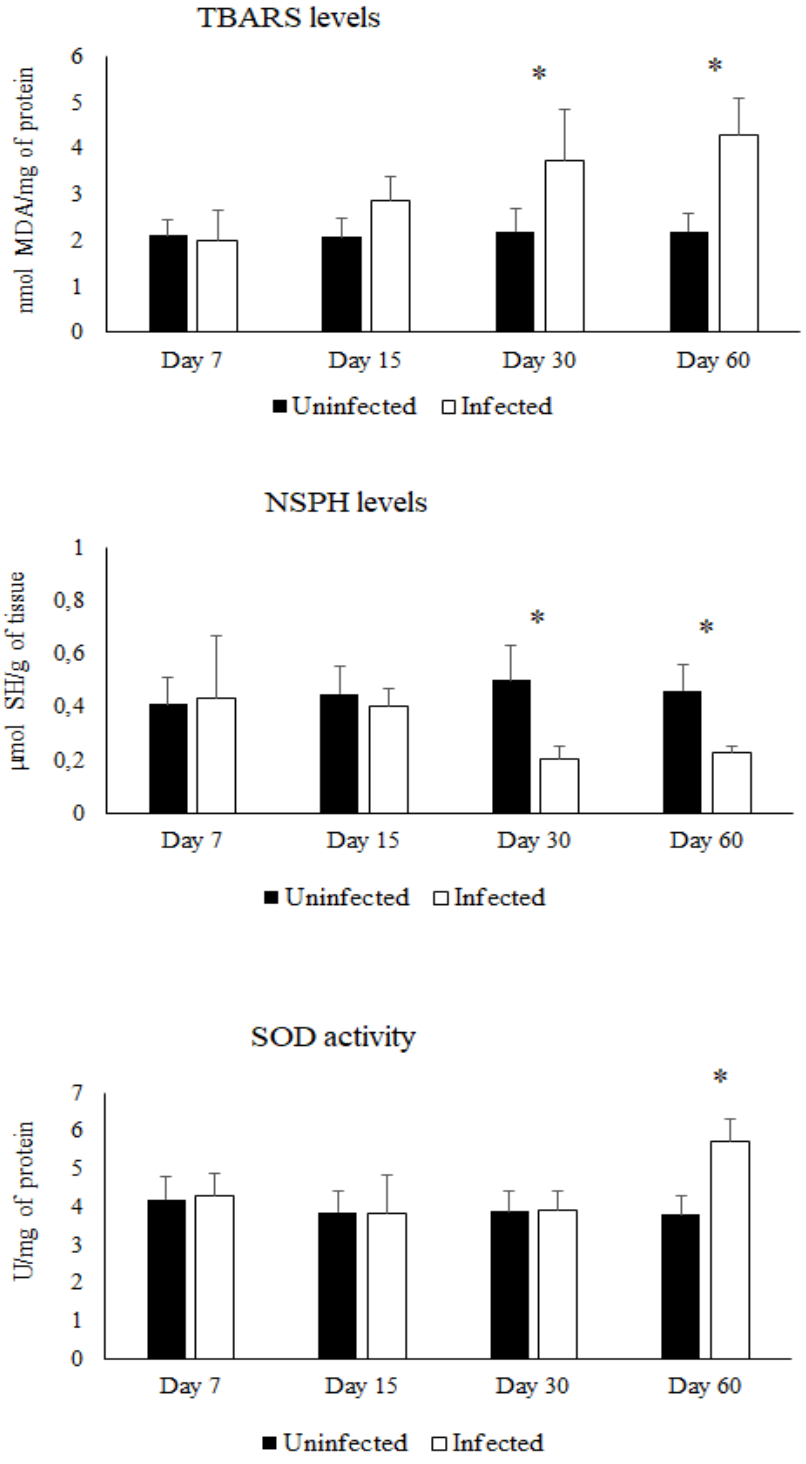

Figure 2: Cardiac thiobarbituric acid reactive substances (TBARS) and non-protein thiol (NPSH) levels, and superoxide dismutase (SOD) activity of mice experimentally infected with *Brucella ovis* on days 7, 15, 30 and 60 post-infection (PI). **P*<0.01; ***P*<0.05.

3 – CONSIDERAÇÕES FINAIS

A importância da brucelose é inquestionável, apesar da *B. ovis* não ter caráter zoonótico como a *B. abortus*. Ambas as espécies dessa bactéria quando presentes nos rebanhos causam grandes perdas econômicas, assim como devem ser combatidas e controladas, principalmente a *B. abortus*. Desta forma, desenvolveram-se estudos que buscaram esclarecer a patogênese destes agentes e a resposta imune dos hospedeiros, sendo alvo de interesse em nossa pesquisa, visando conhecer outros mecanismos envolvidos, que não os tradicionais, na patogenia da doença.

A infecção natural por *B. abortus* mostrou ser capaz de induzir o estresse oxidativo em fêmeas gestantes, o que pode contribuir para o aborto de algumas vacas, visto que, os oxidantes podem causar dano endotelial, aumentando o comprometimento da vascularização e a deficiência imunológica. Em humanos, existem diversas descrições sobre o papel do estresse oxidativo na gestação e sua relação direta com alterações fetais e neonatais (FURNESS et al., 2011), visto que, o estresse oxidativo causa disfunção vascular na placenta (HUBEL, 1999) e o baixo aporte de oxigênio, e assim leva a uma diferenciação anormal do trofoblasto, resultando em diminuição do fluxo sanguíneo placentário e dano ao tecido fetal (GRANGER et al., 2002; MYATT, 2006). Apesar do tipo de placenta ser diferente entre humanos e ruminantes, não pode ser descartado o mecanismo similar do estresse oxidativo nas vacas. Além disso, alterações da atividade de enzimas purinérgicas é um mecanismo compensatório que visa diminuir o processo inflamatório, devido à ação anti-inflamatória da molécula adenosina, desencadeado pela doença e, consequentemente, o dano tecidual que pode provocar o aborto. De acordo com literatura, a placenta é particularmente vulnerável ao estresse oxidativo devido a sua extensa divisão celular e alta atividade metabólica (BURTON et al., 2003; JAUNIAUX et al., 2006).

A infecção experimental por *B. ovis* por sua vez, mostrou-se capaz de gerar alterações hematológicas, bioquímicas e patológicas características da doença em ovinos, portanto, os camundongos foram excelentes modelos experimentais. A resposta inflamatória exacerbada e o aumento dos níveis de EROs contribuem para o dano celular e tecidual provavelmente. Além disso, as alterações enzimáticas no sistema purinérgico e colinérgico e metabolismo energético a nível sérico e tecidual podem influenciar mecanismos inflamatórios, neurológicos e funcionais dos diferentes tecidos alvos da pesquisa, influenciando assim na fisiopatogenia da doença. E ainda, o desencadeamento de um quadro de estresse oxidativo foi demonstrado pelo aumento da produção de espécies reativas ao oxigênio e consumo dos mecanismos antioxidantes gera efeitos extremamente deletérios para

a saúde animal. Importante enfatizar que pesquisas envolvendo coração e cérebro em infecções por *B. ovis* são raras, e em nosso estudo observou-se que esses dois importantes órgãos são afetados bioquimicamente, assim como alterações histológicas cerebrais foram vistas.

De modo geral, todos objetivos propostos foram alcançados, uma vez que, visavam pesquisa básica a fim de conhecer consequências da brucelose experimental por *B. ovis* e natural por *B. abortus*, pontos importantes para futuras estratégias de diagnóstico e controle. Conclui-se também, que prevalência para *B. abortus* em vacas é muito baixa, sendo que em nosso estudo nenhum animal foi soropositivo. No entanto, no estado existem várias propriedades com diagnóstico para doença, sendo monitoradas por órgão estadual competente.

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UDESC UNIVERSIDADE DO ESTADO DE SANTA CATARINA | LAGES CENTRO DE CIÊNCIAS AGROVETERINÁRIAS

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Atividade da adenosina desaminase associado estresse oxidativo em vacas soropositivas para Brucella abortus", protocolada sob o CEUA nº 7688080916, sob a responsabilidade de **Aleksandro Schafer da Silva** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade do Estado de Santa Catarina (CEUA/UDESC) na reunião de 26/05/2017.

We certify that the proposal "Adenosine deaminase activity associated oxidative stress in seropositive cows to Brucella abortus", utilizing 20 Bovines (20 females), protocol number CEUA 7688080916, under the responsibility of **Aleksandro Schafer da Silva** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the University of Santa Catarina State (CEUA/UDESC) in the meeting of 05/26/2017.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 09/2016 a 12/2016 Área: Zootecnia

Origem:	Animais de proprietários						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	3 a 5 anos	N:	20
Linhagem:	Holandesas			Peso:	400 a 600 kg		

Resumo: A brucelose bovina é uma doença causada principalmente pela bactéria Brucella abortus, que quando diagnosticada em propriedades leva ao descarte de animais e restringe comercializações de subprodutos pela enfermidade ter caráter zoonotico. Como o descarte é o destino de animais positivos, pouco se sabe sobre a patogenia doença relacionada a saúde do animal. A adenosina deaminase (ADA) tem mos-trado uma marcador enzimático de resposta inflamatória em várias doenças, assim como estresse oxidativo tem potencializado a sintomatologia de doenças infectocon - tagiosas. Portanto, o objetivo desse estudo foi avaliar a ocorrência de alteração na atividade da ADA e estresse oxidativo em 10 vacas soropositivas para brucelose e comparado 10 vacas soronegativas de uma mesma propriedade no município de São Domingos. Esses animais foram selecionados após coletada de amostras de sangue pela veterinária municipal de 55 vacas e encaminhadas a CIDASC para fins de diagnostico. Amostras de sangue e soro desses animais estão armazenados congelados (-20ºC). Nessas amostras será analisado a atividade da ADA, assim como níveis de peroxidação lipídica (TBARS) e antioxidantes (CAT e SOD). Esse projeto não prevê manipulação dos animais, apenas processar amostras de soro e sangue já coletadas. A proprietária da fazenda que será usado o soro e sangue dos animais está de acordo, assim como assinou o TCLE (em anexo ao projeto).

Local do experimento: Não seria experimento, mas sim local de origem das amostras a serem usadas nesse estudo. Em um propriedade no município de São Domingos, Estado de Santa Catarina, Brasil, vacas apresentavam histórico de aborto em terço final de gestação, bem como repetições constantes de cio.

Lages, 22 de junho de 2017

Marcia Regina Pfuetzenreiter
Coordenadora da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina

Prof. Dr. Ubirajara Maciel da Costa
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina

UDESC UNIVERSIDADE DO ESTADO DE SANTA CATARINA | LAGES CENTRO DE CIÊNCIAS AGROVETERINÁRIAS

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Estresse oxidativo e enzimas purinergicas em camundongos infectados com Brucella ovis", protocolada sob o CEUA nº 4438310517, sob a responsabilidade de **Aleksandro Schafer da Silva** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade do Estado de Santa Catarina (CEUA/UDESC) na reunião de 19/06/2017.

We certify that the proposal "Oxidative stress and purinergic enzymes in mice infected with Brucella ovis", utilizing 48 Heterogenics mice (48 males), protocol number CEUA 4438310517, under the responsibility of **Aleksandro Schafer da Silva** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the University of Santa Catarina State (CEUA/UDESC) in the meeting of 06/19/2017.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 07/2017 a 02/2019 Área: Zootecnia

Origem:	Biotério UNOCHAPECÓ						
Espécie:	Camundongos heterogênicos	sexo:	Machos	idade:	60 a 150 dias	N:	48
Linhagem:	linhagem BALB/c			Peso:	25 a 40 g		

Resumo: A brucelose ovina é causada pela Brucella ovis e apresenta ampla distribuição mundial, sendo considerada uma das principais causas de infertilidade, o que resulta em significativa perda econômica para a ovinocultura devido a problemas reprodutivos e descarte precoce dos animais, além de prejuízos para a produção de lã, carne, leite. Sabe-se que entre os mecanismos fisiológicos, a formação de radicais livres ocorre como mediadores de reações bioquímicas, mas sob condições patológicas, como é o caso da brucelose, pode haver uma superprodução desses radicais, causando danos às células e aos tecidos e, portanto, exigindo a ativação do sistema antioxidante. Nestas mesmas condições, é enfatizada a importância de estudos sobre as enzimas que atuam como marcadores inflamatórios, como é o caso da adenosina deaminase (ADA). Portanto, o objetivo da presente proposta consiste em avaliar se há ocorrência de estresse oxidativo e alteração na atividade de ADA em camundongos infectados por B. ovis. Então, serão usados 48 camundongos nesse estudo, os quais serão divididos em grupo A (n=24; controle) e grupo B (n=24; infectados experimentalmente com Brucella ovis). Nos tempos 05, 10, 30 e 90 dias após a infecção, seis de animais de cada grupo serão anestesiados em uma câmara com isoflurano para colheita de sangue por punção cardíaca (2 mL). A atividade de ADA, NTPDase, 5' nucleotidase (enzimas purinergicas) serão medida espectrofotometricamente em soro e cérebro, enquanto indicadores de estresse oxidativo (CAT, SOD, EROs, TBARS) serão estimados no soro, sangue total, cérebro e baço. Analise histopatológica de baço e cérebro serão feitos.

Local do experimento: Sala de experimentação animal, em anexo ao Laboratório de Anatomia e Fisiologia Animal (sala 5), prédio da Zootecnia, Chapecó.

Lages, 22 de junho de 2017

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