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Experimental Infection by *Brucella ovis*: Changes in NTPDase, 5′-Nucleotidase and Acetylcholinesterase Associated Cerebral Oxidative Stress

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ABSTRACT

***Background*:** Changes in purinergic and cholinergic signaling have been demonstrated in various pathologies associated with inflammation; however, the changes in brucellosis caused by the Gram-negative coccobacillus *Brucella ovis* are not known. *B. ovis* is generally asymptomatic in ewes. Hepatosplenomegaly has been described in *B. ovis*, a non-zoonotic species, characterized by an extravascular inflammatory response. Purinergic system enzymes are closely involved with the modulation of the immune system, pro- and anti-inflammatory events. The objective of this study was to investigate the role of ectonucleotidases and cholinesterase's in the brains of mice experimentally infected with *B. ovis*.

***Materials, Methods & Results*:** Forty-eight animals were divided into two groups: control (n = 24) and infected (n = 24). In group infected, 100 μL containing 1.3 x 10^7 UFC *B. ovis* /mL via intraperitoneal was used in inoculation. The brains were collected from the animals on days 7, 15, 30 and 60 post-infection (PI). We measured 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. 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 in the cerebral cortex were observed at day 60 PI in the infected animals. *B. ovis* DNA was detected in brain. During the course of infection, *B. ovis* caused greater lipid peroxidation in the brains of infected animals than in the control group 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 than in the control group. NTPDase activity (using ATP and AMP as substrate) was lower at days 7 and 15 PI in infected animals than in control. 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. AChE activity in brain on days 30 and 60 PI compared to control.

***Discussion*:** Among the functions of NTPDase are inhibition of platelet aggregation, vascular homeostasis, modulation of inflammation and immune response, all via its regulation of extracellular concentrations of ATP, a pro-inflammatory molecule. E-NTPDase plays an important role in controlling lymphocyte function, including antigen recognition and activation of cytotoxic T cell effector functions, as well as the generation of signals. 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. Levels of acetylcholine are regulated by cholinesterase enzymes that are present in cholinergic and noncholinergic tissues, as the acetylcholinesterase (AChE) is a membrane-bound enzyme, primarily found in the brain and cholinergic neurons, where it participates in the structural regulation of postsynaptic differentiation. The results demonstrated that the chronicity of infection by *Brucella ovis* causes oxidative damage in the brain, as well as modulation of ectonucleotidases and AChE activities.

Keywords: ATP, brucellosis, NTPDase, 5′-nucleotidase, oxidative stress.

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INTRODUCTION

Ovine brucellosis caused by the Gram-negative coccobacillus *Brucella ovis* are not known. *B. ovis* is generally asymptomatic in sheep. An experimental model systemic infection demonstrated lesions in various tissues [18]. Hepatosplenomegaly has been described in *B. ovis*, a non-zoonotic species [15,18], characterized by an extravascular inflammatory response. Our hypothesis is that the bacteria, directly or indirectly, change cholinergic and purinergic signals in the brain of mice, because these systems have a role in regulating the inflammatory response.

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 [2, 4]. 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 [20]. Already, the role of the cholinergic system in the CNS is well documented. Acetylcholine (ACh) is the neurotransmitter of cholinergic synapses and neuroeffector junctions responsible for the formation of nervous impulses, generation and modulation of movement [1, 19]. Free ACh is hydrolyzed by cholinesterase or combines with muscarinic and nicotinic acetylcholine receptors (mAChR and nAChR, respectively) in order to mediate other activities [7]. Therefore, the objective of this study was to investigate the role of ectonucleotidases and cholinesterase's in the brain of mice experimentally infected with *B. ovis*.

MATERIALS AND METHODS

Inoculum

A virulent *B. ovis* strain (ATCC 25840), also known as NCTC10512 or 63/290, was used in the present study. The inoculum preparation was performed following the methodology described by Perin *et al*. [15].

Animals and samples

Forty-eight frozen mouse brain samples (*Mus musculus*, Swiss lineage; 60 days old; 30 ± 0.5 g) from a previous study [15] were used in the present study. The animals were maintained in cages under controlled temperature and humidity (25ºC, 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^{-1} 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 [15]. Cerebral tissue was collected for measurement of enzymatic, and histopathological analyses. Only on day 60, a cerebellar cortex fragment was collected from three animals of each group for analysis of PCR for *B. ovis* [15].

Sample preparation

Brain 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^{-1}), were evaluated by the Coomassie brilliant blue binding assay. All procedures described above were performed at 4ºC.

Lipid peroxidation

Lipid peroxidation was determined by concentration of malondialdehyde (MDA) [14]. The absorbance was measured at 532 nm. TBARS (thiobarbituric acid) levels were expressed as µmol MDA mg^{-1} protein.

Reactive oxygen species (ROS) levels

Reactive oxygen species were measured by 2'-7'-dichlorofluorescein (DCFH) in brain as previously reported [10]. The samples were incubated with DCFH 1 mM at 27ºC over 30 min. The reaction was read on a fluorimeter (emission 525 nm and excitation 488 nm), and the results were expressed as U DCFH mg^{-1} of protein.

NTPDase and 5'-nucleotidase activities

ATP^1 and ADP^1 hydrolysis were evaluated by E-ATPase total enzyme in brain homogenates as described in a modified method [21]. The reactions were carried out in a medium containing KCl (5 mmol L^{-1}), $CaCl_2$ (1.5 mmol L^{-1}), EDTA (0.1 mmol/L), glucose (10 mmol L^{-1}), sucrose (225 mmol L^{-1}) and Tris-HCl buffer (45 mmol L^{-1}, pH 8.0), in a final volume of 200 µL. The hydrolysis of AMP was evaluated by a method

described previously [5,11]; in a reaction medium containing MgSO4 (10 mmol/L) and Tris-HCl buffer (100 mmol L^{-1}, pH 7.5). The enzyme preparations (0.8-1.2 mg of protein) were pre-incubated at 37 ºC 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.

AChE in brain

AChE activity was measured in brain [8] as previously described [17]. 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 g at 4ºC for 20 min. The supernatants were stored at -30ºC until analysis. Brain protein was adjusted for analysis between 1.4-1.8 µg mL^{-1}.

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[2] (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 brain protein) 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 as µmol AcSCh/h/mg protein.

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.

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). Differences were considered statistically significant when $P < 0.05$ between groups.

RESULTS

During the course of infection, *B. ovis* caused greater lipid peroxidation in the brains of infected animals (Figure 1) 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 (Figure 1) than in the control group.

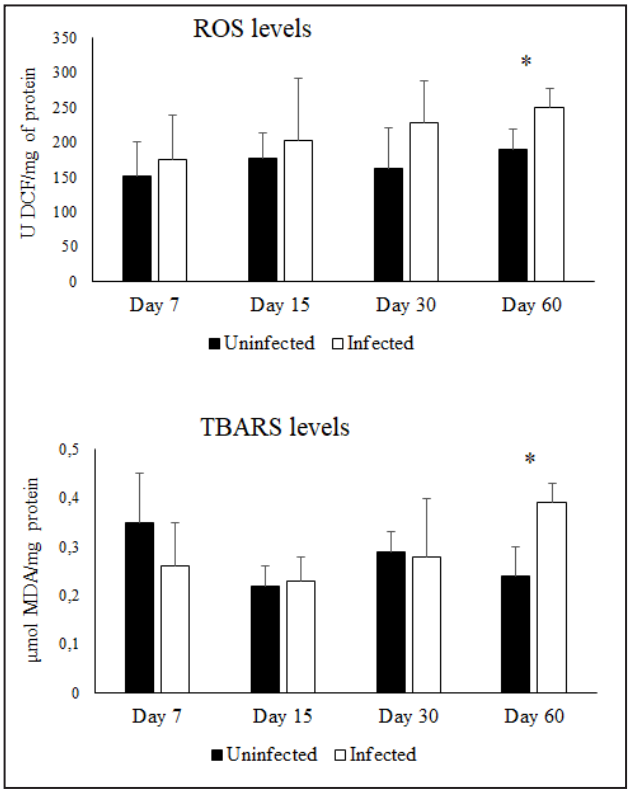

Figure 1. *Brucella ovis* induced oxidative damage during the course of infection. A- Reactive species (RS). B- Substances reactive to thiobarbituric acid (TBARS) of control and infected in mouse brains. Sample collection on days 7, 15, 30 and 60 post infection. The results are expressed as mean ± standard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

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 ($P < 0.05$). 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. AChE activity in brain on days 30 and 60 PI ($P < 0.05$) compared to control (Figure 3).

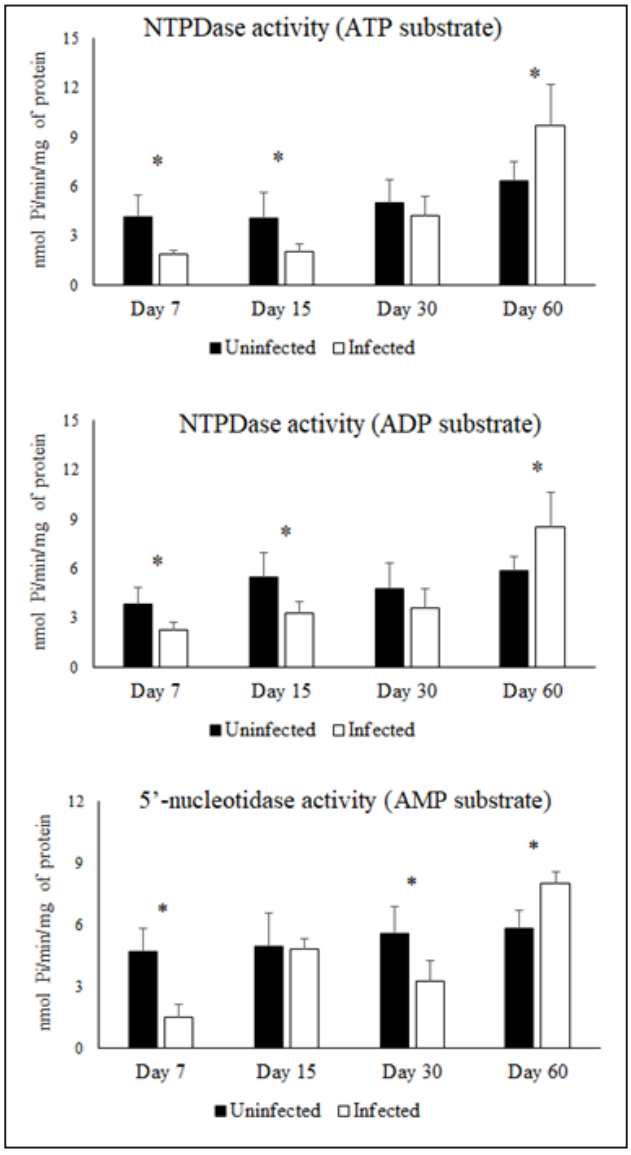

Figure 2. *Brucella ovis* modified activity of ectonucleotidases in brain of infected mice. NTPDase (ATP and ADP) and 5´-nucleotidase (AMP) activities of infected and control groups at days 7, 15, 30 and 60 post infection. The results are expressed as mean ± standard deviation, using the t-test. The results are statistically significant at *P < *0.05*.

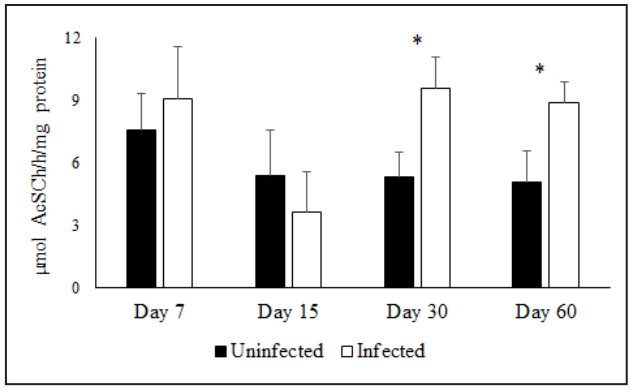

Figure 3. Activity of the enzyme acetylcholinesterase (AChE) in brain. Analysis performed on mice experimentally infected with *Brucella ovis* (n = 12) on days 7, 15, 30 and 60 post-infection (*$P < 0.05$). Results are expressed as the mean ± standard deviation.

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 in the infected animals. *B. ovis* DNA was detected in brain samples analyzed at day 60 PI.

DISCUSSION

The capacity of *B. ovis* to successfully survive and replicate in host cells is critical for its virulence. *Brucella* spp. employ several strategies to establish and maintain persistent intracellular in host cells. The bacterium avoids full-blown inflammatory responses 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 cerebral cortex of mice experimentally infected with *B. ovis*. We found that this infection was associated with multifocal areas of moderate microgliosis with inflammatory infiltrates.

Brucella 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 [9]. 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 [6,16]. *Brucella* spp. strains generate ROS such as O_2^- and H_2O_2 endogenously as a consequence of their aerobic respiratory-type metabolism [12,13]. Exogenous production of these ROS has also been shown to be important for the brucellacidal activity of macrophages [12,13]. In tissues damaged by infections due to a series of inflammatory and oxidative processes, the production of free radicals is high.

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 NTPDase activity during the acute phase of

disease can be considered a pro-inflammatory effect via the reduction of ATP hydrolysis in the extracellular medium [18]. According to these authors, the down-regulation on NTPDase activity by ATP exerts a pro-inflammatory profile due to the augmentation of ATP levels, inducing the release of pro-inflammatory cytokines, consequently contributing to pathophysiology. The increase in NTPDase activity is a direct reflection of the increase in ATP hydrolysis and, consequently, the reduction 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 hydrolysis of the excessive ATP content in the extracellular medium, preventing excessive tissue damage caused by excessive ATP levels during *B. ovis* infection. ATP is an important pro-inflammatory agent involved in vasodilation and platelet activation; it stimulates the production of reactive species, directly stimulating immune cells [3] in order to remove invading pathogens.

The roles of ADP and AMP during inflammation remain unknown. In our study, ADP and AMP hydrolysis was significantly increased in the infected animals at day 60 PI, probably leading to decreases in extracellular concentrations. Augmentation of ADP and AMP hydrolysis can be considered an attempt to produce more adenosine, a molecule with anti-inflammatory and immunosuppressive properties [22].

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 [1], due to increased degradation of ACh by AChE can lead to cerebral dysfunction affecting behavior, memory, locomotion, balance and orientation. According to the literature, the increased AChE activity excessively hydrolyzes ACh in cholinergic synapses and/or neuromuscular junctions, possibly leading to cognitive disorders, behavioral and functional defects related to a hypocholinergic state [1,19].

CONCLUSIONS

Brucella ovis infection in mice causes inflammatory infiltrates in the brain as well as changes in activity of ectonucleotidases and AChE. NTPDase mediates proinflammatory responses during the acute phase and anti-inflammatory with evolution of infection, coinciding with the appearance of lipid peroxidation and increased free radicals in the brain. We also conclude that AChE has a proinflammatory effect in the brain in *B. ovis* infections.

MANUFACTURERS

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

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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