Cholinesterase Activity in Serum, Whole Blood and Lymphocytes of Dogs Experimentally Infected with *Rangelia vitalii*

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**ABSTRACT**

**Background:** The cholinergic system is involved in many biological functions in mammals and is associated with pathogenesis of infectious diseases, as has participation in transmission of nerve impulses in cholinergic synapses, haematopoiesis, regulation of inflammatory markers, production and coordination of movement, and memory. *Rangelia vitalii* is a parasite endemic to south of Brazil. This parasite multiplies in the blood and can be visualized in plasma in its free form and/or within leukocytes and erythrocytes, causing various pathologies. Therefore, the purpose of this study was to investigate the activity of cholinergic system enzymes in dogs experimentally infected with *R. vitalii*.

**Materials, Methods & Results:** Twelve dogs were used, divided into two groups: control group (n = 5), consisting of healthy animals, and infected group with *R. vitalii* (n = 7). Fresh blood samples of these infected animals were inoculated in seven dogs (2 mL/dog through the jugular vein). Blood samples were collected on days 0, 10 and 20 post-infection (PI). Butyrylcholinesterase (BChE) activity was measured in serum and acetylcholinesterase (AChE) in lymphocytes and whole blood. Blood samples were diluted 1:50 (v/v) in lysis solution (0.1 mmol/L potassium/sodium phosphate buffer containing 0.03% Triton X-100) and frozen (-20 °C by 7 days) to determine AChE activity in whole blood. Lymphocytes were also obtained from whole blood with EDTA by gradient separation using Ficoll-Histopaque™ plus to AChE activity this cell. After analysis of the samples, was observed that the dogs infected with *R. vitalii* presented a significant (*P* < 0.01) increase in AChE activity in whole blood on days 10 and 20 PI. However, the infected group showed a reduced activity in AChE in lymphocytes (*P* < 0.01) and BChE in serum (*P* < 0.05) on day 20 PI.

**Discussion:** According to the literature, infected dogs *R. vitalii* develop regenerative anemia evidenced by an increase in the erytroid precursors in bone marrow associated with alterations of leucogram as leukopenia, neutropenia, eosinopenia, lymphocytosis and monocytosis. Furthermore, it was observed severe thrombocytopenia, with alteration in platelet aggregation and activity of enzymes involved in the control of ATP, ADP and adenosine levels on platelets, thereby influencing hemostasis and contributing to the typical bleeding disease. AChE activity in whole blood was increased in dogs parasitized by *R. vitalii* observed in this study. This increase may be a compensatory effect to severe anemia caused by the parasite infection, because this enzyme is involved in the maturation of erythrocytes and in the regulation of hematopoiesis. In the present study, we found that the reduction in AChE activity in lymphocytes is associated to lymphocytosis; and it is known that ACh is produced within lymphocytes and has the ability to negatively modulate the immune response, acting directly on the inhibition of inflammatory mediators. Therefore, the decrease of AChE activity may have an anti-inflammatory action in order to have more free ACh to bind lymphocytes and inhibit inflammation. The enzyme BChE can also act as an inflammatory marker in various diseases, similar to AChE, because the enzyme can hydrolyze acetylcholine when AChE is inhibited. In conclusion, our results indicate that canine rangeliosis alters the activity of cholinesterase’s, which may be involved in the pathogenesis of the disease, as well as various pathological conditions.

**Keywords:** canine, AChE, BChE, rangeliosis.
INTRODUCTION

Acetylcholine (ACh) is a molecule that may affect cell proliferation, differentiation, apoptosis, secretion, cytoskeletal organization, cell–cell cohesion, immune responses and other functions, acting as a signaling molecule [15,24,34]. Regulation of ACh concentrations is regulated by the action of enzymes called cholinesterase’s, including acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) [19,26,32]. AChE is a membrane-bound enzyme mainly found in brain, muscles, erythrocytes, lymphocytes and cholinergic neurons [15,18]; and BChE is found in intestine, liver, kidney, brain, heart, lung and serum [7,8].

*Rangelia vitalii* is a parasite endemic to south of Brazil and with high pathogenicity for dogs [14,17,20], classified as a new species of *Babesia* sp. [28]. This parasite is transmitted by the tick *Amblyomma aureolatum* [27]. Immediately after infection, the parasite multiplies in the blood and can be visualized in plasma in its free form and/or within leukocytes and erythrocytes [6,14,17,20]. Infected dogs may present anemia, jaundice, fever, splenomegaly, generalized lymph node enlargement and persistent bleeding [10,14,20]. Although young animals are more susceptible, adults also develop rangeliosis [14,22]. Canine rangeliosis causes significant alterations in the hematological [12,13]. These physiological alterations may affect cholinergic system enzymes, which are present in blood cells, regulating different biological functions as mentioned previously. Thus, the purpose of this study was to investigate the activity of cholinergic system enzymes in dogs experimentally infected with *R. vitalii*.

MATERIALS AND METHODS

Animals and experimental design

Twelve female mongrel dogs (6-12 months old) were used in this study as previously described [6]. They were divided into two groups: control (n = 5), consisting of healthy animals, and infected with *R. vitalii* (n = 7). The *R. vitalii* isolate used in this study was obtained from a naturally infected dog from Santa Maria, Rio Grande do Sul, Brazil [28] and maintained in living culture (Dog 13) [6]. Fresh blood samples of these infected animals were inoculated in seven dogs (2 mL/dog through the jugular vein), as previously described [6].

Preparation of samples

The animals were contained manually for blood collection of jugular vein (5 mL) on days 0, 10 and 20 post-infection (PI). The storage of the samples was considered according to the analysis. Thus, 4 mL of blood was allocated in tubes containing anticoagulant (EDTA 10%) to separate lymphocytes. The volume of 1 mL was stored in a tube without anticoagulant to obtain serum. These samples were centrifuged for 10 min (3500 g), and the serum was obtained and kept frozen at -20 ºC.

Blood samples 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 whole blood. Subsequently the samples were kept frozen (-20ºC) for 7 days following the methodology previously described [9]. Lymphocytes were also obtained from whole blood with EDTA by gradient separation using Ficoll-Histopaque™ plus, according to the technique described by Böyum [1]. After separation, only samples with at least 95% of lymphocytes, as verified in the STKS counter were used. Lymphocyte viability was confirmed by the Trypan blue dye exclusion and measuring lactate dehydrogenase (LDH) activity [30].

Protein concentration in serum and lymphocytes was determined by the Coomassie blue method [2] using bovine serum albumin as standard.

Acetylcholinesterase activity in whole blood and lymphocytes

AChE enzymatic assay in whole blood was evaluated by the method of Worek *et al.* [36]. The specific activity of AChE was calculated from the quotient between AChE activity and hemoglobin content and the results were expressed as mU/µmol of Hb.

AChE activity in lymphocytes was determined according to the method described by Fitzgerald and Costa [11]. Briefly, proteins of all samples were adjusted to 0.1 - 0.2 mg/mL. Two hundred microliters of intact cells were added to a solution containing 1.0 mM acetylthiocholine (AcSCh), 0.1 mM 5,50-dithiobis (2-nitrobenzoic acid), and 0.1 mM phosphate buffer (pH 8.0). Before and after incubation for 30 min at 27 ºC the absorbance was read on a spectrophotometer at 412 nm. AChE activity in lymphocyte was expressed as µmol of AcSCh/mg of protein.
Butyrylcholinesterase activity in serum

BChE enzymatic assay in serum was determined by a modification of the method of Ellman et al. [9] using the substrate butyrylthiocholine\(^7\) (BcSCh). Samples were pre-incubated at 37°C for 2 min and absorbances were recorded for 2 min at intervals of 20 s on a spectrophotometer at 412 nm. All samples were carried out in duplicate and the enzyme activity was expressed in µmol of BcSCh/h/mg of protein.

Statistical analysis

The results were subjected to the Student’s t-test. Values with probability (\(P\)) less than 5% were considered statistically different. The results were expressed as mean ± standard deviation.

RESULTS

On day 0 PI AChE activity was not altered (\(P > 0.05\)) in whole blood and lymphocytes of infected dogs. On days 10 and 20 PI we observed a significant (\(P < 0.01\)) increase in AChE activity in whole blood of dogs infected with R. vitalii (Figure 1-a). On day 10 PI AChE activity in lymphocytes was not altered (\(P > 0.05\)) in the infected group. On day 20 PI, activity of AChE in lymphocytes was significantly (\(P < 0.01\)) decreased in animals infected (Figure 1-b).

On days 0 and 10 PI BChE activity was not altered (\(P > 0.05\)) in serum of infected dogs. However, on day 20 PI BChE activity was decreased (\(P < 0.05\)) in serum of dogs infected with R. vitalii (Figure 2).

![Figure 1](image_url). Acetylcholinesterase activity in whole blood (A) and lymphocytes (B) of dogs infected with Rangelia vitalii. Asterisk indicates statistical difference between infected and non-infected groups (\(**P < 0.01\)).

**DISCUSSION**

In this study, dogs experimentally infected with *R. vitalii* developed parasitemia and clinical and pathological signs of disease as previously described in detail by our research group [6], similar to the observed in cases of natural infection [10,14,20]. This was the first experimental design which aimed to study the pathogenesis of rangeliosis in different research lines to minimize the number of animals in the study. In the mentioned study it was found that infected dogs develop regenerative anemia evidenced by an increase in the erytroid precursors in bone marrow associated with alterations of leucogram as leukopenia, neutropenia, eosinopenia, lymphocytosis and monocytosis [13]. Furthermore, it was observed severe thrombocytopenia, with alteration in platelet aggregation and activity of enzymes involved in the control of ATP, ADP and adenosine levels on platelets, thereby influencing hemo-stasis and contributing to the typical bleeding disease [21]. In the same study it was described an increase in the markers of lipid peroxidation (TBARS) and protein oxidation (AOPP) in serum and increased activity of antioxidant enzymes such as superoxide dismutase and catalase in blood, which indicate oxidative stress [12]. The aforementioned experimental design also aimed to investigate the cholinergic system profile, since it is involved in several biological functions affected during infection by *R. vitalii*, as discussed below.

AChE and BChE have been linked to the pathogenesis of many diseases, because they are closely linked to the transmission of nerve impulses in cholinergic synapses, haematopoiesis, regulation of inflammatory markers, production and coordination of movement, and memory [5,15,18,24]. Recently, researchers have shown the major influence of infectious diseases on the activity of AChE and BChE in experimental cases of trypanosomosis [35] and leptospirosis [25].

AChE activity in whole blood was increased in dogs parasitized by *R. vitalii* in two periods evaluated in this study. This increase may be a compensatory effect to severe anemia caused by the parasite infection [13], because this enzyme is involved in the maturation of erythrocytes and in the regulation of hematopoiesis [3,18]. In rangeliosis, it has been described some pathological findings suggestive of hemolytic anemia, as a consequence of parasite multiplication within the infected cell [6,10,14]. However, the anemia is regenerative and dogs have a great number of young erythrocytes in circulation [13]. Then, as the red cell membrane is well supplied with AChE, our hypothesis is that the elevation in its activity is directly related to the increased expression of the enzyme in erythrocytes as a response to anemia. Contrary to what occurred in this study, rats infected with *Trypanosoma evansi* showed reduction in AChE activity in whole blood [35]. This may be explained because *R. vitalii* is a parasite of erythrocytic
cycle, unlike trypanosomes. This shows that the pathogenesis of anemia related to cholinesterases activity may differ according to the species of parasite. A study of the expression and characterization of AChE activity in the membrane of erythrocytes could show how this enzyme is involved in the compensatory mechanism of anemia in parasitic diseases.

Infection by *R. vitalii* caused an inhibition of AChE activity in lymphocytes on day 20 PI, which probably is related to the inflammatory response. The existence of cholinergic system in the immune cells is well documented, consisting of acetylcholine muscarinic and nicotinic receptors (mAChR and nAChR, respectively), and enzymes such as choline acetyltransferase (ChAT) and AChE [15,16,31]. In the present study, we found that the reduction in AChE activity in lymphocytes is associated to lymphocytosis as previously described by researchers [13] and reduction of parasitemia [6]. It is known that ACh is produced within lymphocytes [15] and has the ability to negatively modulate the immune response, acting directly on the inhibition of inflammatory mediators [5]. Therefore, the decrease of AChE activity may have an anti-inflammatory action in order to have more free ACh to bind lymphocytes and inhibit inflammation. This inhibition can be explained as a compensatory effect of host to attenuate inflammation and tissue damage caused in the acute phase of illness (Day 10 PI), when parasitemia was high [6].

The enzyme BChE can also act as an inflammatory marker in various diseases, similar to AChE [5], because the enzyme can hydrolyze acetylcholine when AChE is inhibited [19]. In dogs infected with *R. vitalii* it was observed a reduction in BChE activity in serum. This occurred on day 20 PI, when some animals already showed clinical signs such as jaundice and elevated activity of liver enzymes (previously described) [4], which suggests lesion of hepatocytes. Since BChE is synthesized by hepatocytes [33], the liver damage can cause a reduction in its synthesis and/or enzymatic activity. Deficiency of this enzyme may interfere with biological functions, such as regulation of cellular proliferation and neurite growth during development of the nervous system [23,29]. Therefore, the effects observed in BChE activity may directly contribute to the pathogenesis of this disease.

In summary, the experimental infection by *R. vitalii* in dogs causes alteration in the activity of cholinesterases, showing the relationship of the cholinergic system in the pathogenesis of this disease. The increased activity of AChE in blood is directly related to anemia, in an attempt to accelerate the maturation of erythrocytes and decrease the damage generated by hemolytic anemia caused by the piroplasm. In lymphocytes, the reduction in AChE activity may have an anti-inflammatory action in order to reduce the tissue damage observed in this infection. The reduction of BChE in serum possibly occurred as a consequence to liver damage, which affect the synthesis of this enzyme. Therefore, cholinergic system is affected in canine rangeliosis, as well as many other systems already described.

**SOURCES AND MANUFACTURERS**

1. Ficoll-Histopaque™ - Sigma-Aldrich, St. Louis, MO, USA.
2. STKS counter - Bio-rad, Miami, USA.
3. Trypan blue - Sigma-Aldrich, St. Louis, MO, USA.
4. Lactate dehydrogenase - Sigma-Aldrich, St. Louis, MO, USA.
5. Bovine serum albumin - Sigma-Aldrich, St. Louis, MO, USA.
6. Acetylthiocholine - Sigma-Aldrich, St. Louis, MO, USA.
7. Butyrylthiocholine - Sigma-Aldrich, St. Louis, MO, USA.

**Ethical approval.** The procedure was approved (number 15/2010) by the Animal Welfare Committee of Federal University of Santa Maria (UFSM), RS, Brazil.

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

**REFERENCES**


