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Research Brief

Biochemistry detection of acetylcholinesterase activity in *Trypanosoma evansi* and possible functional correlations

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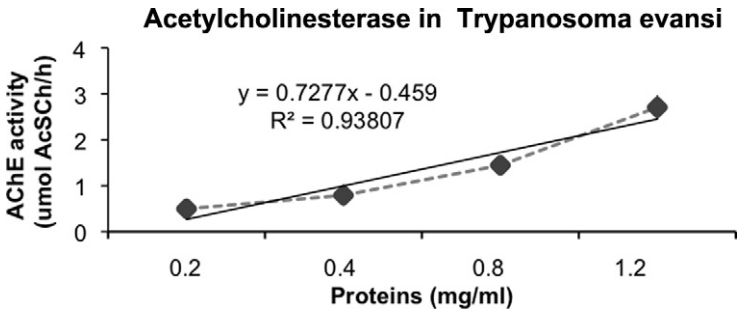
HIGHLIGHTS

- Acetylcholinesterase (AChE) is an important enzyme that hydrolyzes of acetylcholine.
- AChE is broadly distributed in many species, including parasites.
- In the present study we could demonstrate, by biochemistry technical, that the *Trypanosoma evansi* expresses the enzyme AChE.

GRAPHICAL ABSTRACT



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ABSTRACT

Several chemical and immunohistochemical techniques can be used for the detection of acetylcholinesterase (AChE) activity. In this experiment we aimed to detect AChE activity in *Trypanosoma evansi*. For this, the parasites were isolated from the blood of experimentally infected rats using a DEA-cellulose column. Enzymatic activity was determined in trypomastigote forms at 0, 0.2, 0.4, 0.8 and 1.2 mg/mL of protein concentrations by a standard biochemical protocol. At all concentrations tested, the study showed that *T. evansi* expresses the enzyme AChE and its activity was proportional to the concentration of protein, ranging between 0.64 and 2.70 μmol of AcSCh/h. Therefore, we concluded that it is possible to biochemically detect AChE in *T. evansi*, an enzyme that may be associated with vital functions of the parasite and also can be related to chemotherapy treatments, as further discussed in this article.

1. Introduction

Trypanosoma evansi is distributed worldwide, infecting domestic and wild animals (Mahmoud and Gray, 1980; Brun et al., 1998; Silva et al., 1995). This intracellular flagellate can be observed in tissues, blood plasma and body cavity fluids of infected animals leading to an immune response by the host (Sharma et al., 2000). *T. evansi* animals infected develop a range of clinical signs including the increase of body temperature, anemia, loss of physical condition, weakness and neurological dysfunction (Ngeranwa et al., 1993; Wolkmer et al., 2009; Da Silva et al., 2010a). In previous studies, we have demonstrated changes in the cholinergic and purinergic system of rats infected by *T. evansi* (Wolkmer et al., 2010; Da Silva et al., 2010b, 2011a,b). The cholinergic enzyme, acetylcholinesterase (AChE: EC 3.1.1.7) is involved

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during neural disease processes and as a part of the host immune response (Wolkmer et al., 2010; Da Silva et al., 2010a; Paim et al., 2011).

The main role of AChE is termination of transmission at cholinergic synapses by rapid hydrolysis of acetylcholine (ACh) (Rosenberry, 1975; Kimura et al., 2003). The action of AChE is not only limited to cholinergic transmission. It has potent effects on cellular adhesion (Johnson and Moore, 1999), neural extension (Layer et al., 1993; Layer and Willbold, 1994) and structural regulation of postsynaptic differentiation (Soreq and Seidman, 2001). The existence of cholinergic anti-inflammatory pathway is well documented (Tayebati et al., 2002; Kawashima and Fujii, 2003; Kimura et al., 2003; Pavlov and Tracey, 2006; Das, 2007). This mechanism is mediated by ACh acting to inhibit the production of tumor necrosis factor, interleukin-1 and macrophage migration inhibitory factor (Borovikova et al., 2000). Signaling of cholinergic anti-inflammatory pathway occurs through the efferent vagus nerve and is initially mediated by nicotinic acetylcholine receptors (nAChR) on tissue macrophages, which stimulate anti-inflammatory response. Also, AChE is present in lymphocytes regulating immune functions (Kawashima and Fujii, 2003).

Non-neuronal ACh appears to be involved in the regulation of elementary cell functions such as cell mitosis, motion and flagellum activity, as well participating in immune system regulation (Wessler et al., 1998, 2003). The ability of trypanosomes to evade the immune defense mechanism of the host becomes one of the major and puzzling issues in the studies of host–parasite interactions (Zambrano-Villa et al., 2002). The presence of the receptor and some metabolic changes that occur in the parasite after cholinergic stimulation has been reported by Portillo et al. (2011) who demonstrated the presence of nAChR and Mijares et al. (2011) who localized the presence of AChE in glycosomes of *T. evansi*. Acetylcholine has also been detected in many unicellular organisms (including ciliated protozoa). The ubiquity of the cholinergic pathway in the animal kingdom could be part of a mechanism to access intracellular Ca^{2+} via activation of phospholipase C activity, increasing Ca^{2+} concentration (Kimura et al., 2003; Portillo et al., 2011). AChE is involved in multifaceted activities, and serves as a suitable candidate for diagnostic purposes, vaccine development and drug design.

Several chemical and immunohistochemical techniques can be applied for the detection of AChE activity. Biochemical techniques are quick and practical. In this experiment we aimed to measure the AChE activity in *T. evansi* using a biochemical protocol. In addition, we discussed some possibilities of the interaction between the presence of this enzyme in the parasite and the potential relationship with the host.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide, 5,5′-dithio-bis-2-nitrobenzoic acid, tris-(hydroxymethyl)-aminomethane GR, Coomassie brilliant blue G were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in the experiments were of analytical grade and of the highest purity.

2.2. Trypanosome strains

Two rats were inoculated intraperitoneally with 0.2 mL of cryopreserved blood containing 10^6 *T. evansi* per microliter. The parasitemia was estimated daily by microscopic examination of blood smears (Da Silva et al., 2009; Wolkmer et al., 2009). Each slide was mounted with blood collected from the tail vein, stained by the panoptic method, and visualized by optical microscopy. After the 5th day of infection the parasitemia was greater than 100 trypanosomes per microscopic field (1000×). At this time the animals were anesthetized for blood collection, and samples were stored in tubes with anticoagulant.

2.3. Isolation of trypomastigotes forms

Immediately after sample collection trypomastigotes were isolated by chromatography on a Poly-Prep® column (Bio-Rad Laboratories, Hercules, USA) using DEAE–cellulose resin, according to the technique described by Tavares et al. (2010). The number of parasites purified was measured by counting in a Neubauer chamber. In order to further concentrate the parasites, the samples (2 mL of phosphate-buffered-saline-glucose: PSG + parasite) were centrifuged for 30 min (14,000*g* at 4 °C). The pelleted trypanosomes were resuspended in Hanks' balanced salt solution (HBSS).

2.4. AChE activity in the parasite

AChE activity in *T. evansi* was obtained by adapting the technique described by Ellman et al. (1961) and modified by Fitzgerald and Costa (1993) used to evaluate the AChE activity in lymphocytes. Briefly, 0.2 mL of samples were added to a solution containing 1.0 mM acetylthiocholine (AcSCh), 0.1 mM, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) and 100 mM phosphate buffer (pH 8.0). Immediately before and after incubation for 30 min at 27 °C the absorbance was read on a spectrophotometer at 412 nm. As negative and positive controls HBSS and human lymphocytes were used respectively. Peripheral lymphocytes were isolated using Ficoll Hypaque density gradient as described by Böyum, (1998). AChE was calculated from the quotient between trypanosome AChE activity and protein content and the results were expressed as μmol of AcSCh/h and μmol of AcSCh/h/mg of protein. The experiment was replicated twice and the samples were measured in triplicate.

2.5. Protein determination

Protein concentration of the trypomastigotes was determined by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as a standard. Protein concentrations of the samples were adjusted to provide 0, 0.2, 0.4, 0.8 and 1.2 mg/mL.

2.6. Ethics committee

This study was approved by the Ethics and Animal Welfare Committee of the Rural Science Center of the Federal University of Santa Maria (CCR/UFSM), Nº. 017/2012 in accordance with existing legislation and the Ethical Principles published by the Brazilian College of Animal Experiments (COBEA).

3. Results and discussion

In this study AChE enzyme was detected biochemically in *T. evansi*. The biochemical method used to evaluate the activity of AChE in lymphocytes through hydrolysis of the neurotransmitter acetylcholine (Fitzgerald and Costa, 1993), proved to be effective for quantifying the enzymatic activity in the parasite. Fig. 1 shows that increased protein concentration (parasite) resulted in a higher enzymatic activity ($R^2 = 0.91807$). The mean concentration measured was 2.132 ± 0.3 μmol of AcSCh/h/mg of protein, demonstrating that, at all protein concentrations used, the degradation activity of the enzyme was very similar. The reaction did not occur when

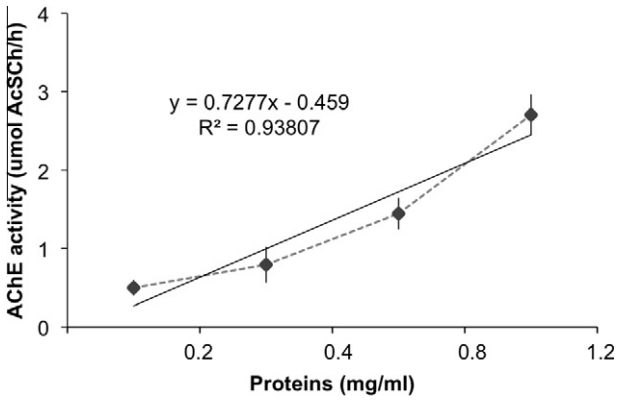

Fig. 1. Biochemical detection of acetylcholinesterase (AChE) in trypomastigotes forms of *T. evansi*. Relationship between AChE activity and protein concentration of the parasite ($n = 9$).

only HBSS (negative control) was used but enzyme activity was observed when lymphocytes were used (positive control).

AChE is an enzyme broadly distributed in many species, including parasites. Initially it was believed that this enzyme was present only in forms that show motion, such as motile protozoa and hydrozoa, but not in non-motile protozoa or sponges (Seaman and Houlihan, 1951; Karczmar, 2010). The authors suggested that the presence of AChE could be correlated with the motility, since, in nerve activity, the action current is controlled by hydrolysis of ACh by AChE. So, the enzyme is fundamental to the regulation of impulse propagation and thus responsible for flagellar and cilia motion. Researches have however shown that the cholinergic system is also present in non-motile organisms, such as plasmodium and the sponges. In the other hand, there is no evidence that AChE is related to flagellar motion of *Physarum polycephalum* (Jahn and Bovee, 1965). Taking this information together, the possibility arises of a non-neuronal role played by the cholinergic system.

Using a standardized protocol to assess the activity of the enzyme (Ellman et al., 1961 modified by Fitzgerald and Costa, 1993) we were able to detect biochemically the presence of AChE in *T. evansi,* this enzyme has also been demonstrated by ELISA recently (Mijares et al., 2011). Probably, in the parasite, this enzyme is responsible for the regulation of ACh levels. Furthermore, *T. evansi* have other components of cholinergic system including nAChR (Portillo et al., 2011). Within the cell, these receptors mediate activation of phospholipase C activity, resulting in increases in [Ca+] and inhibition of adenylyl cyclase activity, resulting in decreased cyclic AMP (cAMP) formation (Hulme et al., 1990). Due to its crucial biological role, AChE is the target of a wide repertoire of drugs and poisons, both natural and synthetic. These include organophosphate insecticides and antihelmintics that act as powerful covalent inhibitors of AChE (Levi-Schaffer et al., 1984; Stieger et al., 1986; Soreq and Seidman, 2001; Harder, 2002a,b). nAChR has a wide distribution in the animal kingdom, and its presence in *T. evansi* widens further its distribution in protozoa (Wessler et al., 1998). Cholinergic stimulation of the nAChR is terminated by hydrolysis of ACh) by the enzyme. In future studies, it is important to demonstrate ACh within the parasite, as well as the presence of choline acetyltransferase (ChAT) enzyme, which is responsible for the synthesis of ACh from acetyl coenzyme A and choline.

Considering the cholinergic anti-inflammatory pathway, if AChE in *T. evansi* was released under stimulation, the enzyme could be acting as modulator of the host immune system. We have previously demonstrated alteration of host-AChE activity during parasitic infections (Da Silva et al., 2010a, 2011a; Wolkmer et al., 2010). Parasite-enzymes could interfere with the course of infection by regulation of the immune response to the parasite associated with immune response evasion. The cholinergic system plays a role in the neuro-immune network of mammals with a high degree of integration between cells of the immune system and the cholinergic system (Rinner et al., 1995). This mechanism is regulated by neural signals transmitted via vagus nerve that suppresses the pro-inflammatory cytokine release, specifically via α7 subunit-containing nicotinic acetylcholine receptor (α7nAChR) (Kawashima and Fujii, 2003). ACh acts directly on macrophage and lymphocyte receptors to open a channel in the cell membrane. Binding to ACh results in reduced nuclear factor (NF-kB) activation, preservation of HMGB1 nuclear localization reduced production of inflammatory cytokines (Das, 2007). Therefore, ACh is partially regulated by autonomic nervous system, and this pathway regulates the release of tumor necrosis factor (TNF), interleukin-1 (IL-1) and other pro- and anti-inflammatory cytokines from immune cells (Elenkov et al., 2000; Tracey, 2002; Pavlov et al., 2003; Pavlov and Tracey, 2004). Whereas, ACh is an anti-inflammatory molecule (Das, 2007) and parasite AChE could interfere on the levels of ACh on host, changing local and systemic inflammatory events caused by trypanosomes infection, thereby regulating the immune response to the parasite.

In conclusion, the present study evidence that *T. evansi* expresses the enzyme acetylcholinesterase, which probably regulates the concentration of acetylcholine, as it does in mammals. The technique used proves to be adequate for detecting AChE biochemically in the parasite.

References

Borovikova, L.V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G.I., Watkins, L.R., Wang, H., Abumrad, N., Eaton, J.W., Tracey, K.J., 2000. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 405, 458–462.

Böyum, A., 1998. Isolation of mononuclear cells and granulocytes from human blood, isolation on mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1*g*. Scand. J. Clin. Lab. Invest. 97, 77–89.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Brun, R., Hecker, H., Lun, Z.R., 1998. *Trypanosoma evansi* and *T. equiperdum*: distribution, biology, treatment and phylogenetic relationship (a review). Vet. Parasitol. 79, 95–107.

Da Silva, A.S., Costa, M.M., Wolkmer, P., Zanette, R.A., Faccio, L., Gressler, L.T., Dorneles, T.E., Santurio, J.M., Lopes, S.T., Monteiro, S.G., 2009. *Trypanosoma evansi*: hematologic changes in experimentally infected cats. Exp. Parasitol. 123, 31–34.

Da Silva, A.S., Spanevello, R., Stefanello, N., Wolkmer, P., Costa, M.M., Zanette, R.A., Lopes, S.T., Santurio, J.M., Schetinger, M.R., Monteiro, S.G., 2010a. Influence of *Trypanosoma evansi* in blood, plasma, and brain cholinesterase of experimentally infected cats. Res. Vet. Sci. 88, 281–284.

Da Silva, A.S., Pierezan, F., Wolkmer, P., Costa, M.M., Oliveiro, C.B., Tonin, A.A., Santurio, J.M., Lopes, S.T., Monteiro, S.G., 2010b. Pathological findings associated with experimental infection by *Trypanosoma evansi* in cats. J. Comp. Pathol. 142, 170–176.

Da Silva, A.S., Pimentel, V.C., Jaques, J.A., Wolkmer, P., Tavares, K.C., Lazzarotto, C.R., Miletti, L.C., Schetinger, M.R., Mazzanti, C.M., Lopes, S.T., Monteiro, S.G., 2011a. Biochemical detection of adenosine deaminase in *Trypanosoma evansi*. Exp. Parasitol. 128, 298–300.

Da Silva, A.S., Monteiro, S.G., Goncalves, J.F., Spanevello, R., Oliveira, C.B., Costa, M.M., Jaques, J.A., Morsch, V.M., Schetinger, M.R., Mazzanti, C.M., Lopes, S.T., 2011b. Acetylcholinesterase activity and lipid peroxidation in the brain and spinal cord of rats infected with *Trypanosoma evansi*. Vet. Parasitol. 175, 237–244.

Das, U.N., 2007. Acetylcholinesterase and butyrylcholinesterase as possible markers of low-grade systemic inflammation. Med. Sci. Monit. 13, RA214–RA221.

Elenkov, I.J., Wilder, R.L., Chrousos, G.P., Vizi, E.S., 2000. The sympathetic nerve – an integrative interface between two supersystems: the brain and the immune system. Pharmacol. Rev. 52, 595–638.

Ellman, G.L., Courtney, K.D., Andres Jr., V., Feather-Stone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Fitzgerald, B.B., Costa, L.G., 1993. Modulation of muscarinic receptors and acetylcholinesterase activity in lymphocytes and in brain areas following repeated organophosphate exposure in rats. Fundam. Appl. Toxicol. 20, 210–216.

Harder, A., 2002a. Chemotherapeutic approaches to schistosomes: current knowledge and outlook. Parasitol. Res. 88, 395–397.

Harder, A., 2002b. Chemotherapeutic approaches to trematodes (except schistosomes) and cestodes: current level of knowledge and outlook. Parasitol. Res. 88, 587–590.

Hulme, E.C., Birdsall, N.J., Buckley, N.J., 1990. Muscarinic receptor subtypes. Annu. Rev. Pharmacol. Toxicol. 30, 633–673.

Jahn, T.L., Bovee, E.C., 1965. Movement and locomotion of microorganisms. Annu. Rev. Microbiol. 19, 21–58.

Johnson, G., Moore, S.W., 1999. The adhesion function on acetylcholinesterase is located at the peripheral anionic site. Biochem. Biophys. Res. Commun. 258, 758–762.

Karczmar, A.G., 2010. Cholinesterases (ChEs) and the cholinergic system in ontogenesis and phylogenesis, and non-classical roles of cholinesterases – a review. Chem. Biol. Interact. 187, 34–43.

Kawashima, K., Fujii, T., 2003. The lymphocytic cholinergic system and its contribution to the regulation of immune activity. Life Sci. 74, 675–696.

Kimura, R., Ushiyama, N., Fujii, T., Kawashima, K., 2003. Nicotine-induced Ca2+ signaling and down-regulation of nicotinic acetylcholine receptor subunit expression in the CEM human leukemic T-cell line. Life Sci. 72, 2155–2158.

Layer, P.G., Weikert, T., Alber, R., 1993. Cholinesterases regulate neurite growth of chick nerve cells in vitro by means of a non-enzymatic mechanism. Cell Tissue Res. 273, 219–226.

Layer, P.G., Willbold, E., 1994. Cholinesterases in avian neurogenesis. Int. Rev. Cytol. 151, 139–181.

Levi-Schaffer, F., Tarrab-Hazdai, R., Meshulam, H., Arnon, R., 1984. Effect of phosphonium salts and phosphoranes on the acetylcholinesterase activity and on the viability of *Schistosoma mansoni* parasites. Int. Immunopharmacol. 6, 619–627.

Mahmoud, M.M., Gray, A.R., 1980. Trypanosomiasis due to *Trypanosoma evansi* (Steel, 1885) Balbiani, 1888. A review of recent research. Trop. Anim. Health Prod. 12, 35–47.

Mijares, A., Concepcion, J.L., Vielma, J.R., Portillo, R., 2011. Immune detection of acetylcholinesterase in subcellular compartments of *Trypanosoma evansi*. Parasitol. Res. 108, 1–5.

Ngeranwa, J.J., Gathumbi, P.K., Mutiga, E.R., Agumbah, G.J., 1993. Pathogenesis of *Trypanosoma* (*brucei*) *evansi* in small East African goats. Res. Vet. Sci. 54, 283–289.

Paim, F.C., Duarte, M.M., Costa, M.M., Da Silva, A.S., Wolkmer, P., Silva, C.B., Paim, C.B., Franca, R.T., Mazzanti, C.M., Monteiro, S.G., Krause, A., Lopes, S.T., 2011. Cytokines in rats experimentally infected with *Trypanosoma evansi*. Exp. Parasitol. 128, 365–370.

Pavlov, V.A., Tracey, K.J., 2004. Neural regulators of innate immune responses and inflammation. Cell. Mol. Life Sci. 61, 2322–2331.

Pavlov, V.A., Tracey, K.J., 2006. Controlling inflammation: the cholinergic anti-inflammatory pathway. Biochem. Soc. Annu. 34, 1037–1040.

Pavlov, V.A., Wang, H., Czura, C.J., Friedman, S.G., Tracey, K.J., 2003. The cholinergic anti-inflammatory pathway: a missing link in neuroimmunomodulation. Mol. Med. 9, 125–134.

Portillo, R., Bruges, G., Delgado, D., Betancourt, M., Mijares, A., 2011. *Trypanosoma evansi*: pharmacological evidence of a nicotinic acetylcholine receptor. Exp. Parasitol. 125, 100–105.

Rinner, I., Felsner, P., Falus, A., Skreiner, E., Kukulansky, T., Globerson, A., Hirokawa, K., Schauenstein, K., 1995. Cholinergic signals to and from the immune system. Immunol. Lett. 44, 217–220.

Rosenberry, T.L., 1975. Acetylcholinesterase. Adv. Enzymol. Relat. Areas Mol. Biol. 43, 103–218.

Seaman, G.R., Houlihan, R.K., 1951. Enzyme systems in *Tetrahymena geleii* S. II. Acetylcholinesterase activity; its relation to motility of the organism and to coordinated ciliary action in general. J. Cell. Physiol. 37, 309–321.

Sharma, D.K., Chauhan, P.P., Agrawal, R.D., 2000. Interaction between *Trypanosoma evansi* and *Haemonchus contortus* infection in goats. Vet. Parasitol. 92, 261–267.

Silva, R.A., Arosemena, N.A., Herrera, H.M., Sahib, C.A., Ferreira, M.S., 1995. Outbreak of trypanosomosis due to *Trypanosoma evansi* in horses of Pantanal Matogrossense, Brazil. Vet. Parasitol. 60, 167–171.

Soreq, H., Seidman, S., 2001. Acetylcholinesterase – new roles for an old actor. Nat. Rev. Neurosci. 2, 294–302.

Stieger, A., Cardoso de Almeida, M.L., Blatter, M.C., Brodbeck, U., Bordier, C., 1986. The membrane-anchoring systems of vertebrate acetylcholinesterase and variant surface glycoproteins of African trypanosomes share a common antigenic determinant. FEBS Lett. 199, 182–186.

Tavares, K.C., Da Silva, A.S., Wolkmer, P., Monteiro, S.G., Miletti, L.C., 2010. Cryopreservation of *Trypanosoma evansi* after DEAE–cellulose purification: evaluation of infective parameters. Res. Vet. Sci. 90, 257–259.

Tayebati, S.K., El-Assouad, D., Ricci, A., Amenta, F., 2002. Immunochemical and immunocytochemical characterization of cholinergic markers in human peripheral blood lymphocytes. J. Neuroimmunol. 132, 147–155.

Tracey, K.J., 2002. The inflammatory reflex. Nature 420, 853–859.

Wessler, I., Kilbinger, H., Bittinger, F., Unger, R., Kirkpatrick, C.J., 2003. The non-neuronal cholinergic system in humans: expression, function and pathophysiology. Life Sci. 72, 2055–2061.

Wessler, I., Kirkpatrick, C.J., Racke, K., 1998. Non-neuronal acetylcholine, a locally acting molecule, widely distributed in biological systems: expression and function in humans. Pharmacol. Ther. 77, 59–79.

Wolkmer, P., Da Silva, A.S., Traesel, C.K., Paim, F.C., Cargnelutti, J.F., Pagnoncelli, M., Picada, M.E., Monteiro, S.G., Lopes, S.T., 2009. Lipid peroxidation associated with anemia in rats experimentally infected with *Trypanosoma evansi*. Vet. Parasitol. 165, 41–46.

Wolkmer, P., Lopes, S.T., Franciscato, C., da Silva, A.S., Traesel, C.K., Siqueira, L.C., Pereira, M.E., Monteiro, S.G., Mazzanti, C.M., 2010. *Trypanosoma evansi*: cholinesterase activity in acutely infected Wistar rats. Exp. Parasitol. 125, 251–255.

Zambrano-Villa, S., Rosales-Borjas, D., Carrero, J.C., Ortiz-Ortiz, L., 2002. How protozoan parasites evade the immune response. Trends Parasitol. 18, 272–278.