Enzymatic activities linked to cardiac energy metabolism of *Trypanosoma evansi*-infected rats and their possible functional correlations to disease pathogenesis

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SUMMARY

The aim of this study was to investigate the activities of important enzymes involved in the phosphoryl transfer network (adenylate kinase and creatine kinase (CK)), lactate dehydrogenase (LDH), respiratory chain complexes and biomarkers of cardiac function in rat experimentally infected by *Trypanosoma evansi*. Rat heart samples were evaluated at 5 and 15 days post-infection (PI). At 5 day PI, there was an increase in LDH and CK activities, and a decrease in respiratory chain complexes II, IV and succinate dehydrogenase activities. In addition, on day 15 PI, a decrease in the respiratory chain complex IV activity was observed. Biomarkers of cardiac function were higher in infected animals on days 5 and 15 PI. Considering the importance of the energy metabolism for heart function, it is possible that the changes in the enzymatic activities involved in the cardiac phosphotransfer network and the decrease in respiratory chain might be involved partially in the role of biomarkers of cardiac function of *T. evansi*-infected rats.

Key words: Phosphotransfer network, cardiac muscle, 'surra', respiratory chain activities.

INTRODUCTION

Trypanosoma evansi is a flagellate protozoan, the etiologic agent of the disease known in Brazil as '*Mal das Cadeiras*' or '*Surra*' in horses (Herrera *et al*. 2004), showing a wide geographical distribution, being found parasiting several domestic and wild animals (Silva *et al*. 2002), and rarely humans (Joshi *et al*. 2005). The parasite is transmitted primarily by blood-sucking insects (*Tabanus* sp., *Chrysops* sp., *Hematopota* sp. and *Stomoxys calcitrans*), as well as possibly by vampire bats (Hoare, 1972). This disease is characterized by rapid weight loss, varying degrees of anaemia, intermittent fever, oedema of the hind limbs and progressive weakness (Herrera *et al*. 2004; Rodrigues *et al*. 2005).

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Studies evaluating cardiac alterations in mice and rats infected by *T. evansi* are scarce, where only histological lesions have been reported (Tejero *et al*. 2010; Bal *et al*. 2012; Finol H. J. and Roschman-González, A. (2014). According to Bal *et al*. (2012) affected myocardium may show degenerative lesions and interstitial oedema; according to Tejero *et al*. (2010) some remarkable signs were observed in mitochondria of cardiac myocytes after 3 days post-infection (PI) such as reduction in the number of mitochondria per cell, decreased dimensionality, and a reduction of the number of mitochondrial cristae.

The heart muscle is highly dependent on mitochondrial energy to provide energy and blood to all organs of the body (Meyer *et al*. 2013) yielding 90% of the cellular energy by generating adenosine triphosphate (ATP;)(Neubauer, 2007). The cardiac energy metabolism has three main components: the first is the use of fatty acids primarily by β-oxidation or glycolysis, using intermediate metabolites of the Krebs cycle (Neubauer, 2007). The second component is oxidative phosphorylation of ADP resulting in ATP production by the respiratory chain.

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ATP is a direct powerful source of energy for all reactions of the heart. The third component is the transfer and use of ATP by myofibrils mediated by enzymes of the phosphotransfer network, particularly creatine kinase (CK; Carvajal and Moreno-Sanchez, 2003; Neubauer, 2007).

The phosphotransfer network is formed by the interaction and complementation between CK, adenylate kinase (AK) and pyruvate kinase (PK), playing a significant role in cellular life by distributing energy and providing metabolic signals required for the coordination of the process in distinct intracellular components (Ingwall, 2004; Saks *et al*. 2006). Studies have reported an association between energy changes mediated by enzymes of the phosphotransfer network related to chronic Chagas' cardiomyopathy (Teixeira *et al*. 2006).

According to Garg *et al*. (2003), mice infected by *Trypanosoma cruzi* have cardiac alteration in the energy metabolism and the oxidative phosphorylation. Additionally, some other authors have reported alterations in the respiratory complexes chain in the heart of mice infected by *T. cruzi* (Vyatkina *et al*. 2004; Baéz *et al*. 2013). In the literature, enzymatic analysis of the respiratory chain complexes' activities localized in mitochondrial cristae is the method most commonly used to confirm suspected defects of mitochondrial energy metabolism (Zheng *et al*. 1990), as we have investigated in our study with *T. evansi*.

Currently, there are very few published data on the cardiac effects of *T. evansi* infections. Histological analysis revealed the presence of trypomastigotes in the vessels of the heart leading to mild degenerative changes in the myocardium followed by interstitial oedema (Bal *et al*. 2012). Biomarkers of cardiac function such as CK, MB-isoenzyme of creatine kinase (CK-MB), lactate dehydrogenase (LDH) and myoglobin have become a major tool for the diagnosis of myocardial necrosis (Jaffe *et al*. 2006). Mitochondrial defects such as energy deficit may lead to cardiac necrosis (Nickel *et al*. 2013).

Therefore, the aim of this study was to evaluate the energy metabolism in heart of *T. evansi* experimentally infected rats in two stages of infection, through the relation between the enzymes of the phosphotransfer network and biomarkers of cardiac function as well as pathological findings to understand the disease.

MATERIALS AND METHODS

Animal model

Twenty-four female rats with 60-days of age weighing 200 (±10 g) on average were used in this study. They were housed in cages on a light/dark cycle of 12 h inside an experimental room with controlled temperature and humidity (23 ± 1 °C; 70%, respectively). Animals were fed with commercial feed and water *ad libitum*. All animals were subject to an adaptation period of 15 days. The *T. evansi* strain was originally isolated from a naturally infected dog (Colpo *et al*. 2005) cryopreserved in liquid nitrogen under laboratorial conditions. This isolate exhibits high pathogenicity to rats, and the animals generally develop acute infection when infected via intraperitoneal (Da Silva *et al*. 2009), therefore the form of infection in rats was subcutaneously, to slow the peak of parasitaemia, as found in the pilot study (unpublished data). Initially, one rat (R_1) was intraperitoneally infected with blood containing 10^6 parasites (0·1 mL). This procedure was performed to obtain large amounts of parasites for this study.

Experimental design and parasitaemia estimation

The animals were divided into four subgroups (A1, A2, B1 and B2) of six animals each. Animals in the subgroups B1 and B2 were inoculated subcutaneously with 0·06 mL of blood from the rat (R_1) containing $6{\cdot}0 \times 10^6$ trypanosomes (day 0).

Animal observation and parasitaemia level by blood smear were daily recorded. Each slide was prepared with fresh blood collected from the tail vein, stained by the panoptic method, and visualized at a magnification of 1000 × according to the method described by Da Silva *et al*. (2006).

The animals were euthanized by decapitation without anaesthetics, and sampling (heart and blood) was performed on days 5 PI (subgroups A1 and B1) and 15 PI (subgroups A2 and B2).

Tissue preparation

Immediately after euthanasia, hearts were removed and dissected on a Petri dish over ice. In order to measure enzymes from the phosphoryl transfer network, the heart was 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 the same SET buffer with a Potter-Elvehjem glass homogenizer. The homogenate was centrifuged at 800 ***g*** for 10 min at 4 °C. Part of this supernatant was 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 supernatant containing cytosol and others cellular components as endoplasmic reticulum was collected for determination of PK, LDH and cytosolic CK activities. The pellet, containing mitochondria was washed twice with the same SET buffer, 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 2 weeks at − 80 °C when the assay was not carried out immediately.

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CK, AK, PK and LDH activities and protein determination

CK activity was assayed in the reaction mixture containing the following final concentrations: 65 mM Tris–HCl buffer, pH 7·5, 7 mM phosphocreatine, 9 mM $MgSO_4$ and 1 μg protein in a final volume of 100 μL. After 10 min of pre-incubation at 37 °C, the reaction was started by the addition of 0·3 μmol of ADP and stopped after 10 min by the addition of 1 μmol of ρ-hydroxymercuribenzoic acid. The concentrations of reagents and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure chemical hydrolysis of phosphocreatine. The creatine was estimated according to the colorimetric method of Hughes (1962). The colour 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 read after 20 min at 540 nm. Results were expressed as nmol of creatine formed per min per mg of protein.

AK activity was measured with a coupled enzymatic assay with hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PD) according to the method of Dzeja *et al*. (1999). 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^{-1} of HK, 2 U mL^{-1} of G6PD and 20 μL of homogenate. The reaction was initiated by the addition of 2 mM ADP and the reduction of $NADP^+$ was followed at 340 nm for 3 min in a spectrophotometer. ADP, $NADP^+$, G6PD and HK were dissolved in milli-Q water. The reagents concentration and assay time (3 min) were chosen to assure the linearity of the reaction. The results were expressed in pmol of ATP formed per min per mg of protein.

PK activity was assayed essentially as described for Leong *et al*. (1981). 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 10 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 phosphoenol pyruvate. All assays were performed in duplicate at 25 °C. Results were expressed as pmol of pyruvate formed per min per mg of protein.

LDH activity was assayed as described by Kaplan *et al*. (1998). The incubation medium consisted of Tris/HCl buffer, pH 7·5, 0·18 mmol L^{-1} NADH, 30 mmol L^{-1} sodium azide and 10 μL of the mitochondria-free supernatant in a final volume of 500 μL. After 5 min of pre-incubation at 37 °C, the reaction was started by the addition of 2 mmol L^{-1} sodium pyruvate. The concentration of the reagents and the incubation time (2 min) were chosen to ensure linearity of the reaction. Enzymatic activity was determined at 340 nm. Results were expressed as pmol of lactate oxidized per min per mg of protein.

Protein content in heart homogenate was determined by the method of Lowry *et al*. (1951) using serum bovine albumin as the standard.

Respiratory chain complexes activities

Mitochondrial respiratory chain enzymatic activities (complexes I–III, II, II–III and IV) were measured in heart homogenates. The activity of NADH: cytochrome *c* oxidoreductase (complexes I–III) was assayed according to the method described by Shapira *et al*. (1990). The activities of succinate : DCIP-oxidoreductase (complex II) and succinate: cytochrome *c* oxidoreductase (complexes II–III) were determined according to Fischer *et al*. (1985) and that of cytochrome *c* oxidase (complex IV) according to Rustin *et al*. (1994). The methods described to measure these activities were slightly modified as described in details in a previous report (Da Silva *et al*. 2002). The activities of the respiratory chain complexes were calculated as $nmol.min^{-1}.mg\ protein^{-1}$.

Biomarkers of cardiac function

Whole blood was collected after animal euthanasia. Total blood and plasma for the analyses of biomarkers of cardiac function were stored in tubes containing ethylenediaminetetraacetic acid. To obtain plasma, samples were centrifuged at 3000 rpm for 15 min and stored at −20 °C until analysis. The plasmatic levels of CK, CK-MB and LDH were evaluated in an automated SBA200 (Celm) by the wet chemistry method. Myoglobin was evaluated in an automated Alere (Triage®MeterPro) by the immunofluorescence method. Tests were carried out in duplicate.

Histology

Heart fragments of uninfected rats and *T. evansi*-infected rats (two animals from each group) were collected and stored in 10% formalin solution. For histopathology, sagittal sections were obtained with an interval of 3 mm between regions and stained with haematoxylin and eosin.

Statistical analyses

The data were tested for normality using the Kolmogorov–Smirnov test, and transformed to logarithm when not normal. Then, determination of the CK, AK, PK and LDH activities, respiratory chain complexes' activities and biomarkers of cardiac function were subjected to Student's *t*-test to

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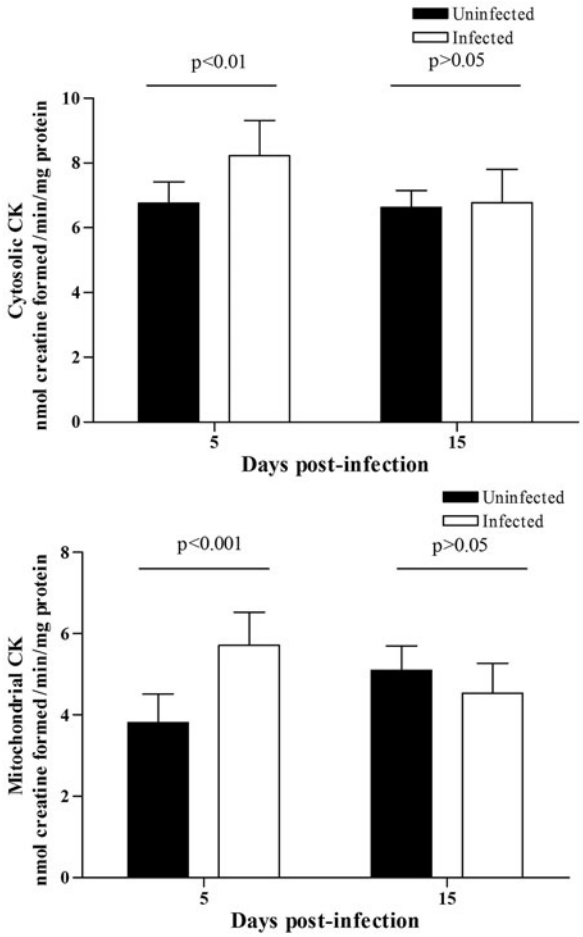

Fig. 1. Average and standard deviation of cytosolic CK and mitochondrial CK activities in rats experimentally infected by *T. evansi* compared with uninfected controls on days 5 and 15 PI. Statistical analysis was performed by Student's *t*-test ($P<0{\cdot}05$).

verify statistical differences between groups and periods (days 5 and 15 PI). Results were considered statistically different with P values $< 0{\cdot}05$.

RESULTS

Disease course

T. evansi was detected in peripheral blood of all infected rats between 48 and 72 h PI. Infected animals showed an average of 14 trypanosomes per field (1000×) at 5 day PI. Fifteen days PI, infected animals had an average of 20 trypanosomes per field (1000×). The control animals remained clinically healthy throughout the experimental period.

CK, AK, PK and LDH activities

The CK activities (cytosolic and mitochondrial) in the heart are shown in Fig. 1. The CK activities increased ($P < 0{\cdot}05$) in the infected group on days 5 PI when compared with the control group. No

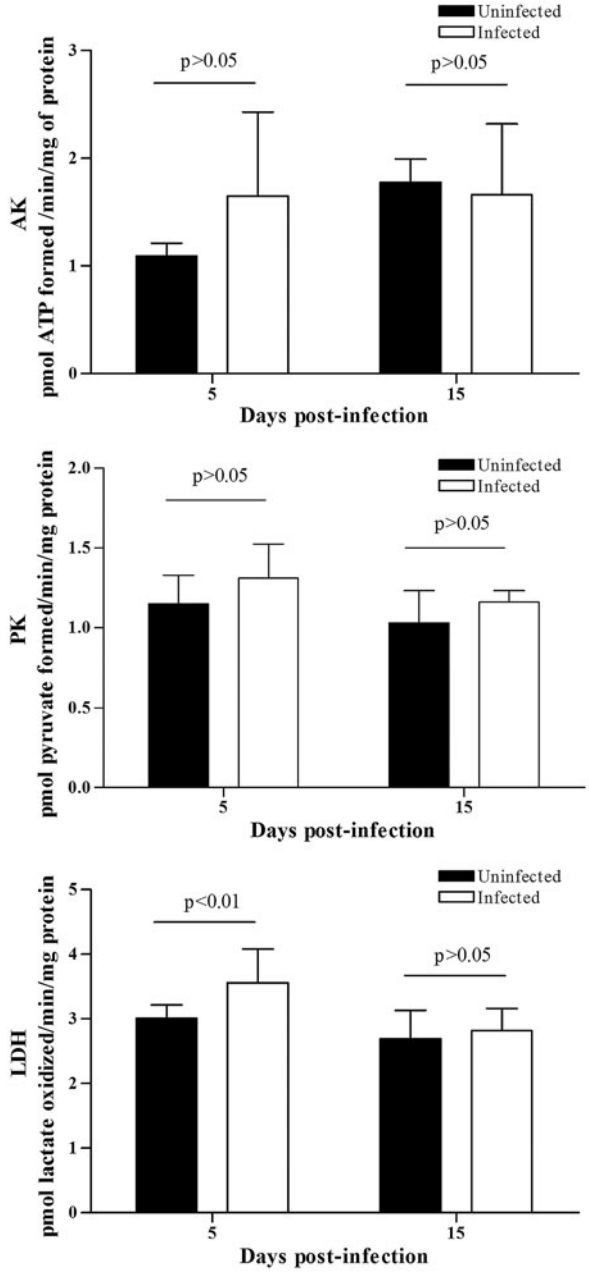

Fig. 2. Average and standard deviation of AK, PK and LDH activities in rats experimentally infected by *T. evansi* compared with uninfected controls on days 5 and 15 PI. Statistical analysis was performed by Student's *t*-test ($P<0{\cdot}05$).

significant difference was observed ($P > 0{\cdot}05$) in the CK activity between days 5 and 15 PI.

The results of AK, PK and LDH activities in the heart are shown in Fig. 2. There were no differences between the groups regarding AK and PK activities ($P > 0{\cdot}05$). Tendency to increase in AK activity in day 5 PI was observed ($P = 0{\cdot}086$). No significant difference was observed ($P > 0{\cdot}05$) in the AK and PK activities between days 5 and 15 PI.

The LDH activity increased ($P < 0{\cdot}05$) in the infected group on day 5 PI compared with the control group (Fig. 2C). It was also observed that

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Table 1. Respiratory chain complex and SDH activities in heart homogenates of *T. evansi* experimentally infected rats on 5 and 15 days PI

Groups	Respiratory chain enzymes activities				
	Complexes I–III	Complex II	Complexes II–III	Complex IV	SDH
5 days PI					
Uninfected	12±3[a]	36±3[a]	33±4[a]	312±55[a]	56±4[a]
Infected	13±2[a]	27±7[b]	32±5[a]	235±34[b]	40±13[b]
15 days PI					
Uninfected	15±4[a]	40±4[a]	34±5[a]	265±46[a]	33±7[a]
Infected	17±3[a]	36±7[a]	33±3[a]	207±31[b]	38±6[a]

Data are expressed as average and standard deviation from six animals per group. The results are expressed as nmol.min^{-1}. mg protein^{-1}. Averages followed by the same letter in the same column are not statistically different by the analysis of Student's *t*-test ($P < 0{\cdot}05$).

Table 2. Effects of *T. evansi* in female Wistar rats on plasmatic biomarkers (CK, CK-MB and LDH) of cardiac function and total blood (myoglobin)

Groups	Biomarkers of cardiac function			
	CK (U L^{-1})	CK-MB(U L^{-1})	Myoglobin(μg L^{-1})	LDH (U L^{-1})
5 days PI				
Uninfected	24·69±6·5[a]	35·45 ± 2·69[b]	5·11±0·22[b]	1319·33±79·72[b]
Infected	21·00±8·24[a]	75·84±11·82[a]	20·83±3·89[a]	1945·16±25·10[a]
15 days PI				
Uninfected	27·66±4·78[b]	37·11±12·33[b]	5·40±0·17[b]	2124·00±87·68[b]
Infected	44·63±13·98[a]	55·00±10·36[a]	20·00±6·73[a]	3224·83±47·94[a]

Data are expressed as average and standard deviation from six animals per group. Averages followed by the same letter in the same column are not statistically different by the analysis of Student's *t*-test ($P < 0{\cdot}05$).

LDH levels were significantly higher ($P < 0{\cdot}05$) on day 5 PI when compared with the day 15 PI the infected rats.

Respiratory chain complex activities

The results of the respiratory chain enzymes activities in heart were shown in Table 1. Complex II (DCIP-oxidoreductase), IV (cytochrome *c* oxidase) and succinate dehydrogenase (SDH) activities were significantly decreased ($P < 0{\cdot}05$) in the infected group on day 5 PI. Complex IV (cytochrome *c* oxidase) activity was significantly decreased in the infected group on day 15 PI.

Biomarkers of cardiac function

The levels of the biomarkers of cardiac function data are shown in Table 2. The CK, MB-CK and LDH activities were quantified in the plasma, and myoglobin was quantified in total blood, in days 5 and 15 PI. The MB-CK and LDH activities and myoglobin were increased ($P < 0{\cdot}05$) in the infected group on day 5 PI when compared with the control group. The CK, MB-CK, LDH activities and myoglobin were increased ($P < 0{\cdot}05$) in the infected group on day 15 PI when compared with the control group.

Histology

The uninfected rats showed no cardiac histological alterations. The animals infected with *T. evansi* showed mild alterations (Fig. 3). On day 5 PI, the presence of acidophilic cytoplasm and nuclei pyknosis inside cardiac cells, as well as necrosis was observed. On day 15 PI, mild multifocal lymphoplasmacytic inflammatory infiltrates were observed.

DISCUSSION

The current study is novel since it evaluates important changes in the phosphoryl transfer network, respiratory chain complexes activities, and biomarkers of cardiac function on experimentally infected rats by *T. evansi*. The analysis of our data clearly shows the stimulation of the cytosolic and mitochondrial CK activities and increases in LDH activity, inhibition of the respiratory chain complexes in the heart and increases in the biomarkers of cardiac

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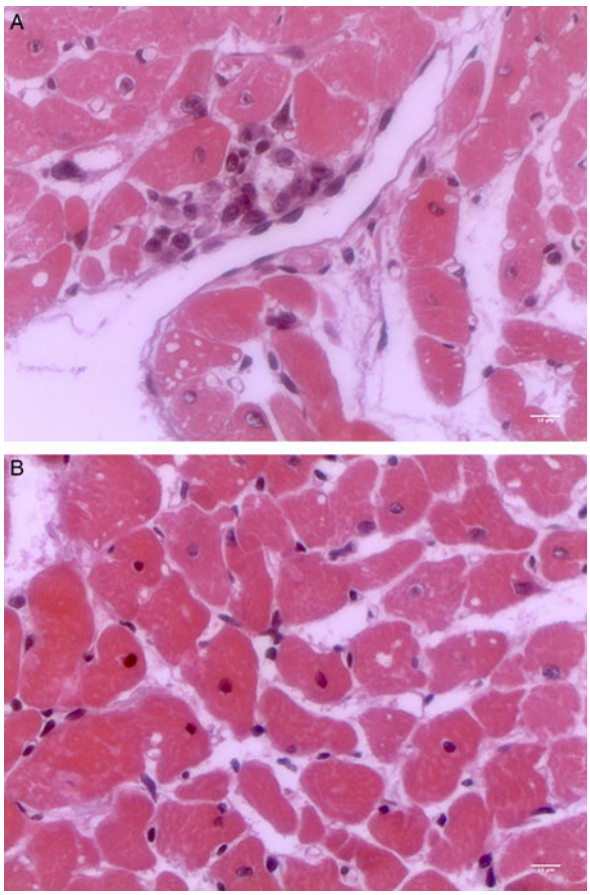

Fig. 3. Heart of rats infected by *T. evansi* showing mild multifocal lymphocytic inflammatory infiltrate (A – Obj: 40×) and acidophilic cytoplasm with pyknotic nuclei (B – Obj: 40×).

function of *T. evansi*-infected rats. The alterations in complex activities were more pronounced on the 5th day PI, whereas in complex II, IV and SDH activities were inhibited throughout the infection. On day 15 PI, it was observed that a decline on the complex IV activity. The decline of respiratory complex activities indicates an imbalance of energy homoeostasis in heart of infected animals.

Metabolic signals regulate and integrate many vital functions in the human body, including energy homoeostasis and heart performance (Dzeja and Terzic, 2009). In cardiomyocytes, production of ATP mainly occurs in mitochondria, which are separated from the ATP consumption sites in the myofibrils (Saks *et al*. 1996). Cells to ensure communication between sites that generate and consume ATP, depend on the phosphotransfer network that facilitates the transfer and distribution of phosphoryls between cellular compartments (Saks *et al*. 1998).

In this study, we showed that *T. evansi* infection was able to increase the enzymatic activity of cytosolic and mitochondrial CK. We emphasize that the one of the functions of the cytosolic CK is to maintain temporal energy, that is, to maintain free ADP in low concentrations and ATP in high concentrations. On the other hand, mitochondrial CK is a modulator of the metabolic potential localized in mitochondria connected to the respiratory chain (Brdiczca *et al*. 1994). Compromised CK function is a hallmark of abnormal bioenergetics in cardiovascular diseases (Ventura-Clapier *et al*. 2010). Reduced CK flux is the most prominent metabolic abnormality related in human heart failure and myocardial infarction (Bottomley *et al*. 2009). Patients diagnosed with Chagas disease, caused by *T. cruzi*, have decreased the expression of muscle CK. However, in our study, we have found different results. When the energy generation exceeds the demand, the level of phosphocreatine increases, reducing the level of free ADP, which could affect homoeostasis (Park and Hourani, 1999), a reason for bleeding already described in the pericardium of cats infected with *T. evansi* (Da Silva *et al*. 2010).

The mitochondrial oxidative phosphorylation system is the final biochemical pathway in the production of ATP. Defects in the oxidative phosphorylation system may result in severe disease (Schapira, 2012).We have observed for the first time, a decrease in the respiratory chain complex activities in heart of rats infected with *T. evansi*. Mitochondria are critical for myocyte function. The contracting cardiac muscle has a high demand of energy that is supplied in the form of ATP produced by mitochondrial oxidative phosphorylation (Chen and Zweier, 2014), and the regulation of oxidative phosphorylation maintains stable the levels of energetic intermediates in order to provide sufficient energy. According to Baéz *et al*. (2013) mice infected with *T. cruzi* show decreased activities of the complexes I and III. It has been previously reported that oxidative stress occurs in proteins in the myocardium of *T. cruzi* infection (Dhiman *et al*. 2012). Complex IV represents the rate-limiting enzyme of the mitochondrial respiratory chain and its activity is an indicator of the oxidative capacity of the cells. This complex is therefore fated to be a central site of regulation of the oxidative phosphorylation, proton pumping efficiency, ATP and reactive oxygen species production, which in turn affect cell signalling and survival (Arnold, 2012).

In vitro and *in vivo* studies indicate that inflammation and reactive species could cause mitochondrial injuries and respiratory chain impairment (Lucas and Szweda, 1999; Sadek *et al*. 2002). *T. evansi*-infected animals show inflammatory process during the infection, evaluated by an increase of pro-inflammatory cytokines such as interferon gamma and tumour necrosis factor in serum (Baldissera *et al*. 2014). Furthermore, rats infected by *T. evansi* develop serum and tissue oxidative stress (Wolkmer *et al*. 2009; Ranjithkumar *et al*. 2011; Baldissera *et al*. 2014). Our data indicate

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a possible mechanism on how *T. evansi* induces mitochondrial injuries, which may lead to the generation of free radicals and to damages in the respiratory chain. The respiratory enzyme complexes are localized in the mitochondrial cristae (Wallace, 1999). *T. evansi*-infected animals had a reduction in the number of mitochondria per cell, a decrease on mitochondrial dimensionality, and a reduction in the number of cristae per mitochondria (Tejero *et al*. 2010). Thus, we believe that these changes in mitochondrial size and number are one of those factors responsible for the inhibition of the respiratory chain complexes.

For the first time we are reporting the effects of *T. evansi* infection on biomarkers of cardiac function and necrosis in the plasma. Infected animals with *T. evansi* showed increased plasmatic levels of CK, CK-MB, LDH and myoglobin in total blood on 5 and 15 days PI (except CK on 5 day PI). According to Dolci and Panteghini (2006), virtually all biomarkers of myocyte necrosis are molecules involved in myocardial contraction or energy production. We have observed an increase on LDH activity in the heart of infected rats on day 5 PI. LDH is a cytosolic enzyme that catalyses the interconversion of pyruvate/NADH and lactate/NAD^+ and it is used as a marker for cell damage, such as muscular necrosis observed in this study (Halliwell and Guteridge, 2007).

Cardiac lesions were detected histologically and biochemically noted as necrosis and inflammatory infiltrates. Mitochondria, besides being the main site of cellular ATP generation, also have a regulatory role in apoptosis and necrosis (Jacobson and Duchen, 2004). Necrosis found on day 5 PI is supported by the increased on LDH activity in heart homogenate and the plasma thus, a marker of muscle necrosis. Several authors have reported that mitochondrial damage, energy metabolism injury and low ATP level are characteristics to necrosis (Scorrano, 2013). According to the literature, sustained ischaemia leads to ATP depletion and subsequently de-energization of the cell, which results in necrotic cell death (Gao *et al*. 2008); therefore during infection by *T. evansi* the ischaemic process may have occurred, since the rats showed cell necrosis, and reduction of respiratory chain complex IV, which will decrease ATP production as described above.

In summary, we have demonstrated for the first time that *T. evansi* infection alters some important parameters of the energy metabolism in rats experimentally infected. Taken into account our present results, it might be suggested that inhibition of the complexes II and IV leads to oxidative damage and to mitochondrial morphological and numerical alterations contribute to the impairment of mitochondrial function in the heart, i.e. decreases in mitochondrial energy production may be implicated in the pathophysiology of the disease.

ETHICS COMMITTEE

The procedures were approved by the Animal Welfare Committee of Universidade do Estado de Santa Catarina under protocol number 01.27.14.

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