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Relationship between pathological findings and enzymes of the energy metabolism in liver of rats infected by *Trypanosoma evansi*

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ABSTRACT

The aim of this study was to investigate the activities of important enzymes involved in the energetic metabolism in the liver of rats experimentally infected by *Trypanosoma evansi*. Adenylate kinase (AK), pyruvate kinase (PK), and lactate dehydrogenase (LDH) in liver homogenate, as well as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and clotting time in plasma were evaluated at 5 and 15 days post-infection (PI). The activities of the respiratory chain complexes and of Na^+, K^+-ATPase were also evaluated. This study demonstrates energetic metabolism impairment in rats infected by *T. evansi*. A reduced energy metabolism in the liver of rats infected by *T. evansi* was observed, demonstrated by AK decreased and PK increased activities at 5 days PI, a mechanism known as energetic compensation. However, at 15 days PI a decrease of AK and PK activities were observed. In addition, an increase in the activities of respiratory chain complexes II, II–III and IV in infected rats at 15 days PI, and a decrease of Na^+, K^+-ATPase activities in infected rats on days 5 and 15 PI were verified. In the plasma, we observed an increase in ALT and AST activities on days 5 and 15 PI, and increase in clotting time in infected rats. The changes caused by *T. evansi* infection on the activity of enzymes of hepatic energy metabolism can corroborate to elucidate the mechanisms that lead to liver injury and inflammatory infiltration verified in *T. evansi* infected rats. Therefore, these alterations are directly related to disease pathogenesis.

1. Introduction

Trypanosoma evansi is the etiologic agent of the disease known in Brazil as "*Mal das Cadeiras*" in horses [1], which may infect several animal species, including domestic and wild animals [2]. This disease is characterized by rapid weight loss, varying degrees of anemia, intermittent fever, edema of the hind limbs, and progressive weakness [1,3]. The liver is one of the most widely affected organs by *T. evansi* [4], leading to changes in oxidative stress parameters [5], aspartate aminostransferase (AST), and alanine aminostransferase (ALT) [6], as well as hepatomegaly and pathophysiological alterations [7].

The hepatic tissue plays a central role in sustaining energetic homeostasis by maintaining a constant supply of energy to fuel bodily tissues [8]. The maintenance of homeostasis between the release and uptake of energy is strictly regulated by intracellular processes. The proper functioning of hepatic bioenergetic system depends on the production and delivery of energetic compounds in the liver, such as adenosine triphosphate (ATP), with a corresponding speed to their rate of consumption [9]. For this, enzymes of energy metabolism formed by adenylate kinase (AK) and pyruvate kinase (PK), act in the transfer of phosphoryl groups between local synthesis and utilization of ATP [10].

AK is the enzyme that catalyzes the phosphotransfer among ATP, adenosine diphosphate (ADP) and adenosine monophosphate (AMP). There are different isoforms of AK (AK1–AK7), whereby the AK2 isoform found in the liver regulates energy metabolism and cell signaling [11,12]. AK is present in the cells and tissues with high energy demand playing an important role in nucleotide synthesis for metabolic functions [13]. Already, PK is an important allosteric enzyme in the glycolytic pathway which catalyzes the transfer of phosphoryl grouping the phospoenolpyruvate (PEP) to ADP to form pyruvate and ATP [14]. In mammals there are four isoforms of PK, L-PK being found in the liver [15].

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Oxidative phosphorylation in the mitochondria provides biological energy for intracellular metabolic pathways. It is particularly significant in hepatocytes because the liver is one of the most energy-consuming organs [16]. Most cell energy is generated through oxidative phosphorylation, a process requiring the well-coordinated action of many respiratory enzyme complexes localized in an inner mitochondrial membrane, the respiratory chain, formed of four complexes (I–IV) [17]. These complexes accept and transfer electrons through oxidation–reduction reactions and translocation of protons across the inner membrane [18]. The proton flux generated during the transport of electrons in the respiratory chain drives the formation of ATP from ADP, and inorganic phosphate through the enzyme ATP synthase (complex V) [18].

Intracellular ion homeostasis in liver cells is accomplished through the concerted action of ion pumps, carriers and channels. Description of these elements includes the sodium potassium pump (Na^+, K^+-ATPase) that is a trans-membrane protein complex found in all higher eukaryotes, acting as a key energy-consuming pump with a vital role of maintaining cell ionic homeostasis. The co-transport of three ions of Na^+ to the intracellular part produces an electrochemical gradient through the cell membrane [19]. This transport is really important since more than one third of the consumed ATP is used to bomb such ions. To the maintenance of the resting membrane potential of tissues, the electrochemical gradient by Na+, K+-ATPase plays a role in several cellular functions including maintenance of the osmotic balance of the cell, which supplies the energy fuels for proper functioning of the cell [20].

Besides increased activity for ALT and AST, the use of other biochemical parameters for a good assessment of liver dysfunction during *T. evansi* infection has not been studied yet. Stands out that lactate dehydrogenase (LDH) is a cytosolic enzyme that catalyzes the interconversion of pyruvate/NADH, and lactate/NAD^+ that are involved in the anaerobic glycolysis and is used as a marker for cell damage [21]. Thus, due to the importance of energy homeostasis to the liver, the aim of this study was to evaluate the activity of important enzymes such as AK, PK, LDH, respiratory chain complexes, and Na^+, K^+-ATPase in liver samples of *T. evansi* experimentally infected rats in two stages of infection, as well as the relationship of these enzymes with biochemistry changes and clotting time, in addition to pathological findings.

2. Materials and methods

The *T. evansi* strain was originally isolated from a naturally infected dog [22], which was maintained cryopreserved in liquid nitrogen under laboratory conditions. This isolate exhibits high pathogenicity to rats, and the animals generally develop acute infection when infected intraperitoneally [23]. Thus, we have used the subcutaneous route to slow the peak of parasitemia, as found in a pilot study (unpublished data). Initially, one rat (R_1) was infected intraperitoneally with blood (cryopreserved in liquid nitrogen) containing 2×10^6 parasites. This procedure was performed to obtain a large number of parasites for this study.

Twenty-four (female) 60-day-old Wistar rats weighing an average of 200 ± 10 g were used as our experimental model. All animals were from the Central Animal Laboratory of the Federal University of Santa Maria (UFSM), Brazil. They were kept in cages, housed on a light/dark cycle of 12 h in an experimental room with controlled temperature and humidity (23 ± 1 °C; 70%, respectively). Animals were fed with commercial ration and water ad libitum. All animals were subject to a period of adaptation of 15 days.

The twenty-four animals were divided into two groups (A and B — 12 animals/each group) and from these again divided into four subgroups (A1 and A2, 6 animals/each group; and B1 and B2, 6 animals/each group). Animals in groups B1 and B2 were inoculated subcutaneously with 0.06 mL of blood from a rat (R_1) containing 6.0×10^6 trypanosomes (day 0).

Rats were observed and parasitemia was monitored daily through blood smears. Each slide was prepared with fresh blood collected from the tail vein, stained by the panoptic method, and visualized at a magnification of 1.000× according to the method described by Da Silva et al. [23].

On day 5 samples from subgroups A1 and B1 were collected as well as for subgroups A2 and B2 on day 15 PI. The animals were sacrificed by decapitation without use of an anesthetic and whole blood and fragments of the liver were collected for analyses. Blood was stored in tubes containing ethylenediamine tetraacetic acid to separate plasma for hepatic function analysis. Clotting time was analyzed with blood collected in vacuum tubes with sodium citrate. The samples were centrifuged at 3000 rpm for 15 min and stored at −20 °C until analysis. Liver fragments were immediately dissected on a glass petri dish over ice. For the measurement of the enzymes of phosphoryl transfer network, liver tissue 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 the supernatant was used for the determination of AK activity; the pellet was discarded and the rest of the supernatant was centrifuged at 10,000 ×*g* for 15 min at 4 °C. The supernatant of this second centrifugation, containing cytosol and other cellular components as endoplasmatic reticulum, was collected for determination of PK and LDH activities.

AK was measured with a coupled enzyme assay with hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PD), according to Dzeja et al. [24]. The reaction mixture contained 100 mM of KCl, 20 mM of HEPES, 20 mM of glucose, 4 mM of $MgCl_2$, 2 mM of $NADP^+$, 1 mM of 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 of 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. Reagent concentration and assay time (3 min) were chosen to assure the linearity of the reaction. The results were expressed in μmol of ATP formed per min per mg of protein.

PK was assayed essentially as described from Leong et al. [25]. The incubation medium consisted of 0.1 M Tris/HCl buffer, pH 7.5, 10 mM of $MgCl_2$, 0.16 mM of NADH, 75 mM of KCl, 5.0 mM of ADP, 7 U of 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 by the addition of 1 mM of phosphoenol pyruvate. All assays were performed in duplicate at 25 °C. The results were expressed as nmol of pyruvate formed per min per mg of protein.

LDH was assayed as described by Kaplan et al. [26]. The incubation medium consisted of Tris/HCl buffer, pH 7.5, 0.18 mmol/L of NADH, 30 mmol/L of 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 of sodium pyruvate. The concentration of the reagents and the incubation time (2 min) were chosen to ensure linearity of the reaction. Enzyme activity was determined at 340 nm. The results were expressed as pmol of lactate oxidized per min per mg of protein.

Protein content in liver homogenates was determined by the method of Lowry et al. [27], using bovine albumin serum as the standard.

Mitochondrial respiratory chain enzyme activities (complexes I–III, II, II–III, and IV) were measured in liver homogenates. The activity of NADH: cytochrome *c* oxidoreductase (complex I–III) was assayed in the liver according to the method described by Shapira et al. [28]. The activities of succinate: DCIP-oxidoreductase (complex II) and succinate: cytochrome *c* oxidoreductase (complex II–III) were determined according to the method of Fischer et al. [29] and that of cytochrome *c* oxidase (complex IV) according to Rustin et al. [30]. The methods described to measure these activities were slightly modified, as described in detail in a previous report by Da Silva et al. [31]. The activities of the respiratory chain complexes were calculated as $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$.