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The Decrease on Na^+, K^+-ATPase Activity in the Cortex, but not in Hippocampus, is Reverted by Antioxidants in an Animal Model of Sepsis

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Abstract In the present study, we investigated whether sepsis induced by cecal ligation and puncture (CLP) modifies Na^+, K^+-ATPase activity, mRNA expression, and cerebral edema in hippocampus and cerebral cortex of rats and if antioxidant (ATX) treatment prevented the alterations induced by sepsis. Rats were subjected to CLP and were divided into three groups: sham; CLP—rats were subjected to CLP without any further treatment; and ATX–CLP plus administration of *N*-acetylcysteine plus deferoxamine. Several times (6, 12, and 24) after CLP or sham operation, the rats were killed and hippocampus and cerebral cortex were isolated. Na^+, K^+-ATPase activity was inhibited in the hippocampus 24 h after sepsis, and ATX treatment was not able to prevent this inhibition. The Na^+, K^+-ATPase activity also was inhibited in cerebral cortex 6, 12, and 24 h after sepsis. No differences on Na^+, K^+-ATPase catalytic subunit mRNA levels were found in the hippocampus and cerebral cortex after sepsis. ATX treatment prevents Na^+, K^+-ATPase inhibition only in the cerebral cortex. Na^+, K^+-ATPase inhibition was not associated to increase brain water content. In conclusion, the present study demonstrated that sepsis induced by CLP inhibits its Na^+, K^+-ATPase activity in a mechanism dependent on oxidative stress, but this is not associated to increase brain water content.

Keywords Sepsis · *N*-acetylcysteine · Deferoxamine · Na^+, K^+-ATPase activity · Brain water content

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Introduction

Sepsis is defined as the hosts' reaction to infection characterized by a systemic inflammatory response [1]. It is a complex syndrome characterized by an imbalance between pro-inflammatory and anti-inflammatory responses to pathogen [2]. The systemic inflammatory response seems to be initiated by the release of bacterial lipopolysaccharide or other microbial substances into the lymphatic and circulatory system. When the sepsis cascade is triggered, an unregulated systemic response that can progress to multiple organ failure occurs [3].

Several molecular mechanisms of inflammation and cellular damage have been implicated in the pathogenesis of sepsis, septic shock, and multiple organ failure, including those related to overt generation of cytokines, eicosanoids, and reactive oxygen species (ROS) [4]. ROS are believed to be important mediators of cellular injury that contribute to the development of sepsis. The pro-inflammatory effects of ROS include endothelial damage, formation of chemotactic factors, neutrophil recruitment, cytokine release, and mitochondrial impairment [5, 6], all contributing to oxidant–antioxidant imbalance.

Na^+, K^+-ATPase (EC 3.6.1.37) is a crucial enzyme responsible for the generation of the membrane potential necessary to maintain neuronal excitability and cellular volume control [7]. The enzyme is present in high concentration in brain cellular membranes and consumes about 40–50 % of the ATP generated [8] being crucial for brain development and function. The inhibition of its activity is found in various diseases, including cerebral ischemia [9, 10] and neurodegenerative disorders [11], and is probably associated with excitotoxicity. A role of the Na^+, K^+-ATPase activity and neurotransmitter release has been demonstrated, suggesting that this enzyme plays a role in the neurotransmission modulation [12, 13].

It is well-described that Na^+, K^+-ATPase is highly vulnerable to free radical inactivation [14], and that oxidative stress plays a crucial role in the development of sepsis [6]. Thus, we hypothesized that sepsis is associated with a decrease in Na^+, K^+-ATPase activity and that antioxidant treatment is able to prevent this alteration in the rat brain.

Materials and Methods

Animals

Adult male Wistar rats (60 days old) were obtained from Universidade do Extremo Sul Catarinense (UNESC, Criciúma, Brazil) breeding colony. They were housed five per cage with food and water available ad libitum and were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.). All experimental procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

Cecal Ligation and Perforation Surgery

The animals were subjected to cecal ligation and puncture (CLP) as previously described [15]. Briefly, rats were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg), given intraperitoneally. Under aseptic conditions, a 3-cm midline laparotomy was performed to allow exposure of the cecum with the adjoining intestine. The cecum was tightly ligated with a 3.0 silk suture at its base, below the ileocecal valve, and was perforated once with a 14-gauge needle. The cecum was then gently squeezed to extrude a small amount of feces from the perforation site, returned to the peritoneal cavity, and the laparotomy was closed with 4.0 silk sutures. All animals received isotonic saline solution (50 mL/kg s.c.) immediately after. All animals were returned to their cages with free access to food and water. In the sham-operated group, the rats were submitted to laparotomy, the cecum was exposed with the adjoining intestine and it was manipulated, but not ligated nor perforated, then it was returned to the peritoneal cavity, and the laparotomy was closed and received isotonic saline solution (50 mL/kg s.c.) immediately after. To minimize the possibility that animals did not truly develop sepsis, the CLP procedure was always performed by the same investigators. In addition, all animals were observed after CLP to determine signs of infection (pyloerection, lethargy, tachypnea, and weight loss), and the number of animals that survived is in accordance with our previous reports [15, 16].

To access parameters in lethal sepsis, as well as the effect of treatments, the animals were divided into three groups: sham—rats were subjected to laparotomy without any other manipulation; CLP—rats were subjected to CLP without any further treatment; CLP + antioxidant (ATX)—rats received NAC (20 mg/kg) 3, 6, 12, and 18 h after CLP plus DFX (20 mg/kg) 3 h after CLP with a subcutaneous injection [15, 16]. The sham and CLP groups were allocated randomly during the procedure. Several times (6, 12, and 24) after CLP or sham operation, six rats were killed by decapitation without the use of sedative, anesthetic, or tranquilizing drugs, and brain structures (hippocampus and cerebral cortex) were immediately isolated and stored at 80 °C. All animals presented signs of encephalopathy at 6 h after sepsis (lethargy, mild ataxia, lack of spontaneous movement, and loss of righting reflex) and gradually returned to their normal awake status 24–36 h after CLP [17].

Tissue Preparation

The hippocampus and cerebral cortex were homogenized in ten volumes (1:10, *w*/*v*) of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.5. The homogenates were centrifuged at 1,000×*g* for 10 min; the supernatants were removed for Na^+, K^+-ATPase activity determination. Protein was measured by the method of Bradford [18] using bovine serum albumin as standard.

Table 1 Primer sequences and PCR amplification conditions

Na^+, K^+-ATPase catalytic subunits	GenBank accession number	Primers (5′–3′)	PCR product	Tm (°C)
Alpha 1	NM_012504	F-TCTATGGACGACCATAAACTCAGCCTGG R-AGCAGACAGCACGACCCCGAGGTAC	297	62
Alpha 2	NM_012505	F-ACCAAGTGGATCTGTCCAAGGGCCTC R-GCTTCCTGGTAGTAGGAGAAGCAGCCAG	292	62
Alpha 3	NM_012506	F-AAAGATGACAAGAGCTCGCCCAAGAAG R-TGATCTCCACCAGGTCCCCGACCAC	538	62
β-actin	NP_742006	F-TATGCCAACACAGTGCTG CTGG R-TACTCCTGCTTCCTGATCCACAT	210	54

Na^+, K^+-ATPase Activity Assay

The reaction mixture for Na^+, K^+-ATPase assay contained 5.0 mM MgCl2, 80.0 mM NaCl, 20.0 mM KCl, and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 μl. After 10 min of pre-incubation at 37 °C, the reaction was initiated by addition of ATP to a final concentration of 3.0 mM and was incubated for 20 min. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays according to the method of Wyse and colleagues [9, 10]. Released inorganic phosphate (Pi) was measured by the method of Chan and colleagues [19]. Specific activity of the enzyme was expressed as nanomoles of Pi released per minute per milligram of protein.

Analysis of Gene Expression by Semiquantitative RT-PCR

The analysis of Na^+, K^+-ATPase catalytic subunits expression was carried out by a semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay. The hippocampus and cerebral cortex were dissected under sterile conditions and immediately subjected to a total RNA extraction by TRIzol® method (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA species were synthesized with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) from 2 μg of total RNA and oligo(dT) primer in accordance with the suppliers. RT reactions were performed for 50 min at 42 °C. cDNA (0.1 mL) was used as a template for PCR with the specific primers for Na^+, K^+-ATPase catalytic subunits. Sequences encoding to each one of Na^+, K^+-ATPase catalytic subunits (alpha 1, alpha 2, and alpha 3) were aligned using ClustalX program. Regions with low scores of similarity among the sequences were used for searching specific primers, which were designed using the program Oligos 9.6. In order to confirm the primer specificity, each primer was compared with rat genome and it was able to recognize only its specific target sequence. Thus, the strategy adopted to construct the primers did not allow cross-amplification (Table 1). β-actin PCR was carried out as an internal standard. PCR reactions were performed with a total volume of 25 μl using a final concentration of 0.08 μM of each primer indicated below, 1.6 mM of $MgCl_2$, and 1 U Taq Platinum Polymerase (Invitrogen) in the supplied reaction buffer. Conditions for Na^+, K^+-ATPase catalytic subunits PCR were as follows: initial 2 min denaturation step at 94 °C; 1 min at 94 °C; 1 min annealing step at 62 °C; 1 min extension step at 72 °C for 30 cycles; and a final 10 min extension at 72 °C. Conditions for *β-actin* PCR were as follows: initial 1 min denaturation step at 94 °C, 1 min at 94 °C, 1 min annealing step at 54 °C, 1 min extension step at 72 °C for 35 cycles, and a final 10 min extension at 72 °C. PCR products were submitted to electrophoresis using 1 % agarose gel with GelRed® (Biotium). The fragments length of PCR reactions was confirmed with Low DNA Mass Ladder (Invitrogen, USA). The relative abundance of each mRNA versus

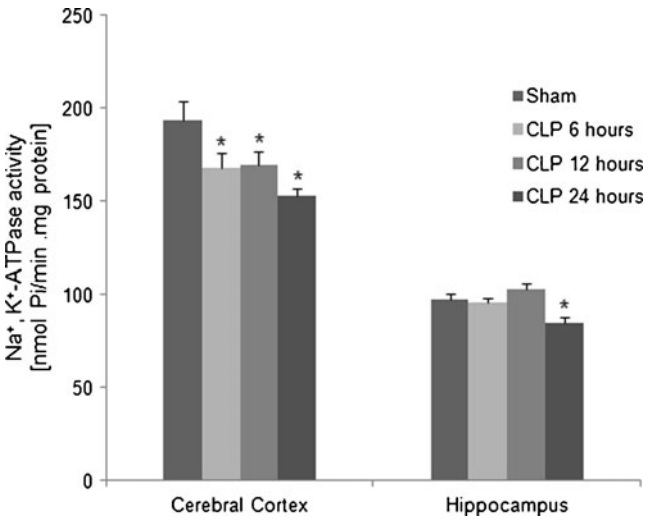

Fig. 1 Na^+, K^+-ATPase activity in cerebral cortex and hippocampus 6, 12, and 24 h after sepsis. Data were analyzed by Duncan's multiple range tests for independent samples and are expressed as mean±standard error of mean. *$P<0.05$ compared to control

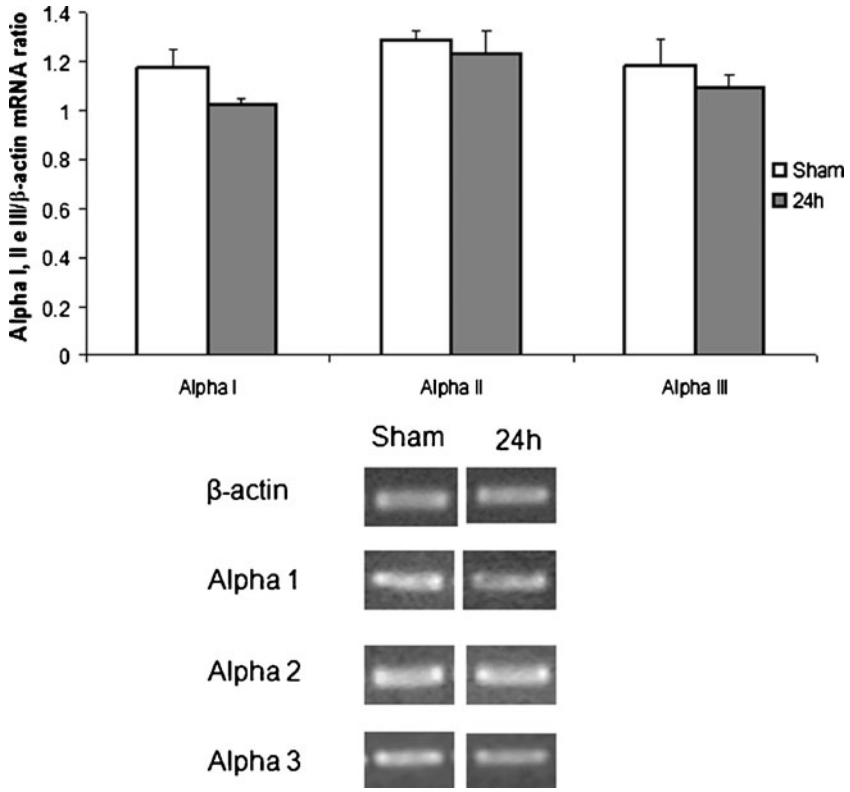

Fig. 2 Na^+, K^+-ATPase catalytic subunit mRNA levels in hippocampus 24 h after sepsis. The PCR products were subjected to electrophoresis on a 1 % agarose gel, using β-actin as constitutive gene. The figure shows a representative gel and the Na^+, K^+-ATPase catalytic subunits/β-actin mRNA ratios (expressed as arbitrary units) obtained by optical densitometry analysis of four independent experiments, with entirely consistent results. Data analyzed by Tukey's HSD post hoc tests for independent samples are expressed as mean±standard error of mean. *$P<0.05$ compared to control

β-actin was determined by densitometry using the freeware ImageJ 1.37 for Windows.

Brain Water Content

Brain edema was evaluated by the drying–weighing method based on the measurement of the water content of brain [20]. The rats were decapitated and cortex and hippocampus were taken. Immediately after being removed, the brain tissue was placed on a filter paper for the removal of the excess water. The porcelain capsule was dried in an incubator. Then, the tare was calculated. Afterward, the brain tissue was placed in the porcelain capsule and weighed. Next, the brain tissue in the porcelain capsule was put in an incubator

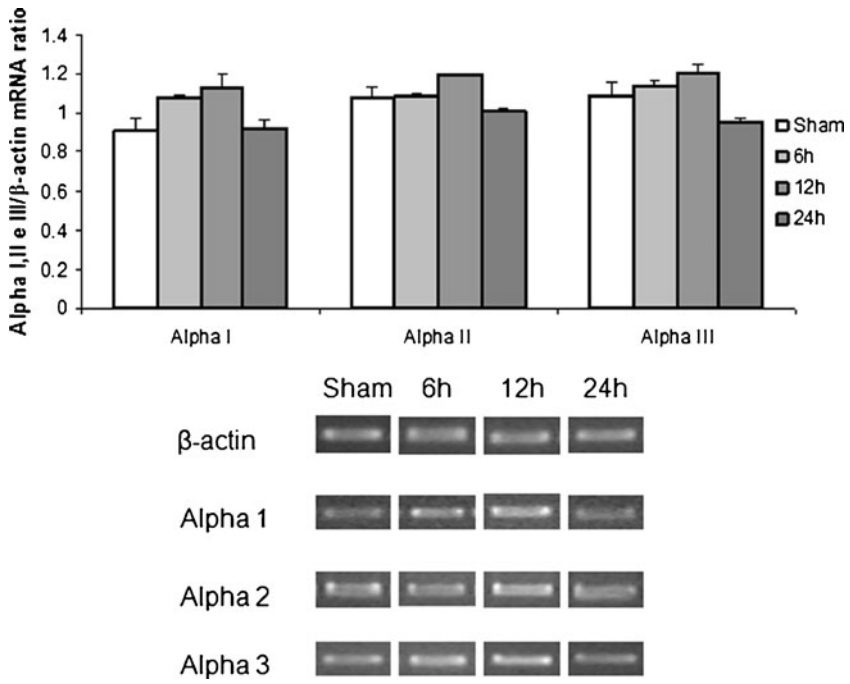

Fig. 3 Na^+, K^+-ATPase catalytic subunits mRNA levels in cerebral cortex 6, 12, and 24 h after sepsis. The PCR products were subjected to electrophoresis on 1 % agarose gel, using β-actin as constitutive gene. The figure shows a representative gel and the Na^+, K^+-ATPase catalytic subunits/β-actin mRNA ratios (expressed as arbitrary units) obtained by optical densitometry analysis of four independent experiments, with entirely consistent results. Data were analyzed by Tukey's HSD post hoc tests for independent samples are expressed as mean±standard error of mean. *$P<0.05$ compared to control

with constant temperature and humidity to be dried for 24 h at 100 °C; 24 h later, the dried brain in the porcelain capsule was reweighed. The percentage of water was calculated according to the following formula: $\%H_2O=[(\text{wet weight}-\text{dry weight})/\text{wet weight}]\times 100$.

Statistical Analysis

Results are presented as means±standard error of mean. Na^+, K^+-ATPase activity and cerebral edema data were analyzed by one-way ANOVA followed by the Duncan's multiple range tests when the F test was significant. Na^+, K^+-ATPase catalytic subunit mRNA levels data were analyzed statistically by one-way ANOVA followed by Tukey's HSD post hoc tests. All analyses were performed using the Statistical Package for the Social Sciences software in a PC-compatible computer. Values of $P<0.05$ were considered significant.

Results

Na^+, K^+-ATPase activity was inhibited in the hippocampus 24 h after sepsis. On the other hand, the Na^+, K^+-ATPase activity was inhibited in cerebral cortex 6, 12, and 24 h after sepsis (Fig. 1). The downregulation of Na^+, K^+-ATPase activity could be a consequence of transcriptional control and/or posttranslational modifications in its catalytic subunits (Table 1). Therefore, we evaluate the catalytic subunit transcripts in the hippocampus and cerebral cortex. There were no differences on Na^+, K^+-ATPase catalytic subunit mRNA levels when compared to controls and septic animals both in hippocampus (Fig. 2) and in cerebral cortex (Fig. 3). These results strongly suggest that the downregulation of Na^+, K^+-ATPase activity in the hippocampus and cerebral cortex of rats submitted to sepsis is a consequence of a posttranslational modification. Moreover, considering that the activity of Na^+, K^+-ATPase is inhibited by oxidative stress, we investigated the effect of ATX treatment on enzyme activity. Our results demonstrate that ATX treatment was able to prevent inhibition of Na^+, K^+-ATPase activity only in the cerebral cortex (Fig. 4).

Since the inhibition of Na^+, K^+-ATPase could be associated with brain swelling, the relation between Na^+, K^+-ATPase activity and brain water content was investigated, but in this model, we could not demonstrate an increase in brain water content both in the cerebral cortex and hippocampus (Table 2).

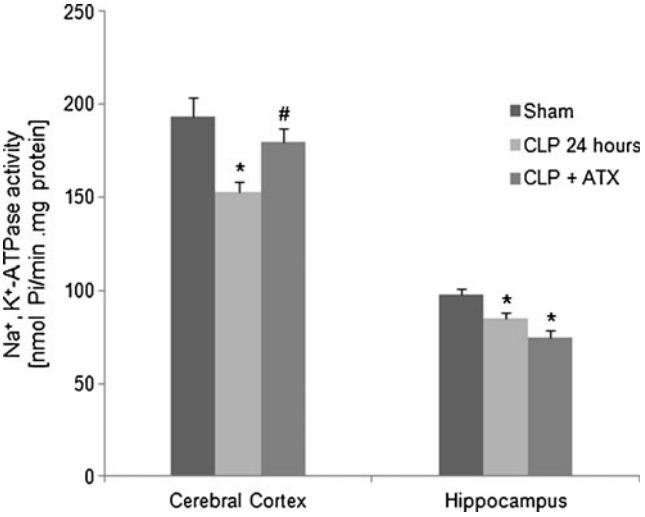

Fig. 4 Effect of antioxidants on Na^+, K^+-ATPase activity in cerebral cortex and hippocampus 24 h after sepsis. Data were analyzed by Duncan's multiple range tests for independent samples and are expressed as mean±standard error of mean. *$P<0.05$ compared to control. *Number sign:* Different from CLP-SAL group ($P<0.05$)

Discussion

In the present study, we demonstrated that Na^+, K^+-ATPase activity is inhibited in the cerebral cortex and hippocampus, but differently from other SNC disease brain water content which was not associated with Na^+, K^+-ATPase inhibition. An extensive body of evidence indicates that sepsis is associated with increased ROS production, depletion of antioxidants, and accumulation of markers of oxidative stress [21]. In the context of sepsis, mitochondrial dysfunction dependent on ROS was demonstrated in the liver [22], heart [23], skeletal muscle [24], and in the brain [25]. In the brain, early oxidative stress has been documented in septic rats, especially in the hippocampus and cortex [5]. Cassol and colleagues [26] demonstrated that sepsis inhibited complex I and II activities, and that ATX treatment was able to prevent this inhibition. In addition, ATX prevented long-term cognitive impairment in septic rats [27]. In this context, Na^+, K^+-ATPase is involved in several physiopathological functions such as regulation of cell volume, cell differentiation, and maintenance of sodium and potassium equilibrium through biological membranes. As regards to the possible consequences of the inhibition of Na^+, K^+-ATPase activity to neural cellular metabolism and function, it should be

Table 2 Water content in hippocampus and cerebral cortex 6 and 24 h after sepsis. Data were analyzed by Duncan's multiple range tests for independent samples and are expressed as mean±standard error of mean

Groups	Hippocampus	Cerebral cortex
Sham	0.8130 (0.0050)	0.7905 (0.0330)
CLP 6 h	0.8158 (0.0195)	0.7946 (0.0012)
CLP 24 h	0.7952 (0.0013)	0.7832 (0.0004)
CLP + ATX	0.8193 (0.0031)	0.7962 (0.0024)

considered that there is increasing evidence suggesting that alterations in Na^+, K^+-ATPase activity may be a link between many common neurotoxic mechanisms in neurons and neuronal death [28]. Furthermore, when Na^+, K^+-ATPase activity is impaired, Na^+ concentration increased intracellularly, collaborating to the pathological mechanisms involved in cerebral edema [29]. In our study, we observed that ATX reestablished Na^+, K^+-ATPase activity, but this was not related with brain edema. Comim and colleagues [30] showed an increase in the permeability of the blood–brain barrier only 24 h after CLP, thus it is possible that in this model, increased brain content of water could be demonstrated only at later times after sepsis and be related to blood–brain barrier breakdown. In addition, other studies indicated that Na^+, K^+-ATPase inhibition activates the apoptotic cascade and neuronal injury probably by amplifying the disruption on potassium homeostasis [31], suggesting that the alterations observed here could be associated with the occurrence of acute and chronic brain dysfunction observed in sepsis.

That Na^+, K^+-ATPase activity has been shown to be dynamically regulated in a number of tissues by hormones and neurotransmitters through activation of second messenger-dependent protein kinases (2–3). It has also been shown that the alpha-1 subunit of Na^+, K^+-ATPase is a substrate for both cAMP-dependent protein kinase and protein kinase C (PKC) in vitro and that the alpha-1 subunit is also phosphorylated by PKC in intact cells [32]. In agreement, recent studies [33] found that PKC phosphorylates the rat alpha-1 subunit of Na^+, K^+-ATPase at Ser-23 in vitro. A large number of studies have provided evidence that Na^+, K^+-ATPase undergoes a series of conformational changes during its normal cycling [34]. In this scenario, studies have demonstrated that phosphorylation of the rat alpha-1 subunit of Na^+, K^+-ATPase occurs on the NH2-terminal cytoplasmic region (Ser-23) and is associated with a change in the conformational equilibrium of the enzyme [34]. Studies suggest that the NH2-terminal region acts to increase Na^+, K^+-ATPase activity and that removal of the region through truncation, or a structural alteration due to phosphorylation, eliminates the ability of this region to activate the enzyme. In any event, it is clear that the NH2-terminus plays an important role in the regulation of enzyme activity [35]. It was observed that rat Na^+, K^+-ATPase catalytic subunits present a high predicted score of possible phosphorylation sites: alpha 1: Ser-23 (PKC), alpha 2: Ser-940 (PKA), and alpha 3: Ser-933 (PKA) according to analysis performed in NetPhosk, a kinase-specific prediction of protein phosphorylation site tool. Therefore, the downregulation of Na^+, K^+-ATPase activity observed after sepsis induced by CLP could be attributed to possible changes in phosphorylation state.

It has been demonstrated that the structural properties and lipid composition of synaptosomal membrane are essential for enzyme activity and that even low concentrations of free radicals inhibit Na^+, K^+-ATPase activity of rat brain [8]. Within this context, it has been observed that Na^+, K^+-ATPase activity is decreased by lipid peroxidation [36, 37] and that –SH groups of cell proteins are highly susceptible to oxidative stress [38]. Corroborating this hypothesis, this protocol of antioxidant treatment prevented the inhibition of Na+, K^+-ATPase activity only cerebral cortex. In this scenario, it is tempting to speculate that one of the mechanisms of inhibition of Na^+, K^+-ATPase activity in the cerebral cortex is possibly by oxidation of fundamental thiol groups in the structure of the enzyme necessary to its function. However, the inhibition of Na^+, K^+-ATPase activity observed in the hippocampus was not reversed by the antioxidant treatment. Studies have reported that apoptosis is an important mechanism during the immunopathogenesis of sepsis [39, 40] and suggested hippocampus as the most vulnerable brain region [41–43]. Thus, the increased apoptosis in hippocampal regions suggest that this particular region may be more vulnerable to inflammatory and/or circulatory changes observed in the brain. It has been previously reported that rats survived and fully recovered 10 days after CLP demonstrated impaired spatial learning and memory [17] and some cognitive skills, such as memory, did not completely improve in most of the sepsis patient at 1-year follow-up [44]. In this context, considering that the antioxidant treatment was not able to prevent the inhibition of Na^+, K^+-ATPase activity in the hippocampus, it is tempting to speculate that this inhibition can be a result of increased apoptotic cell death in this structure.

In conclusion, the present study demonstrated that during sepsis, there is an inhibition of a crucial enzyme of central nervous system which is necessary for maintaining the basal membrane potential necessary for a normal neurotransmission and brain water content. We suggest that the inhibition of Na^+, K^+-ATPase is not associated to brain edema but could induce brain dysfunction during sepsis. In addition, at least in the cerebral cortex, the oxidation of fundamental thiol groups in the structure of the enzyme could be associated with Na^+, K^+-ATPase inhibition.

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