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Aeromonas caviae alters the cytosolic and mitochondrial creatine kinase activities in experimentally infected silver catfish: Impairment on renal bioenergetics

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ABSTRACT

Cytosolic and mitochondrial creatine kinases (CK), through the creatine kinase-phosphocreatine (CK/PCr) system, provide a temporal and spatial energy buffer to maintain cellular energy homeostasis. However, the effects of bacterial infections on the kidney remain poorly understood and are limited only to histopathological analyses. Thus, the aim of this study was to investigate the involvement of cytosolic and mitochondrial CK activities in renal energetic homeostasis in silver catfish experimentally infected with *Aeromonas caviae*. Cytosolic CK activity decreased in infected animals, while mitochondrial CK activity increased compared to uninfected animals. Moreover, the activity of the sodium-potassium pump (Na^+, K^+-ATPase) decreased in infected animals compared to uninfected animals. Based on this evidence, it can be concluded that the inhibition of cytosolic CK activity by *A. caviae* causes an impairment on renal energy homeostasis through the depletion of adenosine triphosphate (ATP) levels. This contributes to the inhibition of Na^+, K^+-ATPase activity, although the mitochondrial CK activity acted in an attempt to restore the cytosolic ATP levels through a feedback mechanism. In summary, *A. caviae* infection causes a severe energetic imbalance in infected silver catfish, which may contribute to disease pathogenesis.

1. Introduction

Aquaculture produces large quantities of fish in a biologically and economically efficient way, since it provides a source of proteins and essential micronutrients for humans [1]. However, infectious diseases, such as that caused by *Aeromonas caviae*, cause direct effects on fish production, leading to severe economic losses for fish producers. This is considered the major impediment to the development of aquaculture [2].

Aeromonas caviae is a facultative anaerobic Gram-negative bacteria belonging to the Aeromonadaceae family; it is found in food, drinking water, sewage and in environmental water, and it possesses the capacity to infect a vast number of hosts, such as humans and fishes [3]. In fish, this disease is characterized by hemorrhagic septicemia, hepatosplenomegaly and eye disease [4], as well as ulcerative lesions on the body surface, bases of the fins and around the anus [5]. This results in high mortality and economic losses in species such as common carp (*Cyprinus carpio*) [6], Indian catfish (*Clarias batrachus*) [5], Nile tilapia (*Oreochromis niloticus*) [7], tambaqui (*Colossoma macropomum*) [8], silver barb (*Barbodes gonionotus*) [9] and rainbow trout (*Oncorhynchus mykiss*) [10]. The kidney is one of the organs most affected by *A. caviae* [5], but the effects of bacterial infection on renal tissue remain poorly understood, and are limited only to histopathological alterations, such as renal liquefaction [4]. Thus, more studies are needed to understand the mechanisms of disease pathogenesis, such as the involvement of creatine kinase (CK), an essential enzyme for the

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maintenance of bioenergetic homeostasis.

Creatine kinase (CK) is an enzyme belonging to the phosphotransfer network, and it catalyzes the reversible transfer of a phosphoryl group from adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and creatine to produce phosphocreatine (PCr). The CK/PCr system plays a storage and distribution role in cellular energetics in areas with a high energy demand, such as the kidney [11,12]. The PCr acts as an energy source, buffer and energy transporter, shuttling energy from subcellular sites (mitochondria) to sites of energy consumption, where the cellular ATPases (facilitating hydrolysis) and ATP-dependent ion pumps are located, such as the sodium-potassium pump (Na^+, K^+-ATPase) [13].

The CK/PCr system is active in kidney epithelial cells, exerting an important role in renal ion homeostasis. The kidneys depend on a variety of ion pumps, such as Na^+, K^+-ATPase. These are energetically demanding and depend on a localized high ATP/ADP ratio, which is guaranteed by the presence of CK and PCr [14]. In this sense, both cytosolic and mitochondrial CK are considered as essential energetic supports for the proper function of Na^+, K^+-ATPase in the renal cells [15]. Amaral et al. [16] demonstrated that downregulation of Na^+, K^+-ATPase activity is linked with impairment of CK activity, contributing to the pathogenesis of diseases. Thus, our hypothesis is that impairment of CK activity leads to a downregulation of renal Na^+, K^+-ATPase activity during an *A. caviae* infection, contributing to energy depletion.

Based on this evidence, the aim of this study was to evaluate the involvement of cytosolic and mitochondrial CK activity in the renal energetic homeostasis in silver catfish, *Rhamdia quelen*, experimentally infected with *A. caviae.*

2. Materials and methods

2.1. Fish harvesting, maintenance and water quality parameters

Healthy fish were collected for experimental purposes from a fish farm located in southern Brazil. The animals were transported to the Laboratório de Fisiologia de Peixes at the Universidade Federal de Santa Maria, where they were maintained in 250 L fiberglass tanks with continuous aeration under controlled water parameters: temperature 18–20 °C (maintained with air conditioner), pH 7.1–7.3 and dissolved oxygen levels 5.9–7.5 mg/L, in freshwater for seven days. Dissolved oxygen and temperature were measured with a YSI oxygen meter (Model 75,512, Ohio, USA), and the pH was measured using a DMPH-2 pH meter (Digimed, São Paulo, Brazil). Total ammonia levels were determined according to Verdouw et al. [17] and non-ionized ammonia levels were calculated according to Colt [18]. The animals were fed to apparent satiation with commercial feed once a day. Any uneaten food, feces and other residues were removed daily 1 h after feeding. The water quality variables were maintained as follows: water temperature 19 ± 1 °C, pH 7.2 ± 0.2, dissolved oxygen 6.43 ± 0.43 mg/L, total ammonia 0.98 ± 0.002 mg/L and non-ionized ammonia 0.006 ± 0.0005 mg/L.

2.2. Inoculum confirmation and preparation

The pathogen was confirmed through colony morphology and physiological characteristics (circular, 2–4 mm in diameter, entire, smooth, convex, cream colored, Gram-negative rods and motile), as well as through biochemical tests: oxidase and catalase positive. The polymerase chain reaction (PCR) also confirmed the pathogen by the analysis of the 16S rRNA gene of *A. caviae* using the primers 5′ TCG TTG GGT TGG GAT GTG 3' (forward) and 5′ TGT TAC CGC GGT GAA AGG 3' (reverse), according to the methodology described in detail by Thomas et al. [5].

The bacterial isolate was grown on nutrient agar for use in this experimental model. The suspension of *A. caviae* was washed twice in sterile saline (NaCl 0.9%), turbidity (OD_{600}) was adjusted to 0.9–1.1 (equivalent to 10^6 CFU/mL) and used for the infection model.

2.3. Animal model and study design

Twenty adult silver catfish (118 ± 21 g; 30 ± 3 cm) were used as the experimental model to assess the renal cytosolic and mitochondrial CK activities, as well as the renal Na^+, K^+-ATPase activity. Fish were assigned into two groups with 10 animals each: uninfected animals (negative control group) and experimentally infected animals (positive control group) inoculated intramuscularly with 100 μL of a bacterial suspension containing 55 × 10^6 viable cells of *A. caviae,* according the protocol established by Thomas et al. [5]. The negative control group received the same dose of sterile saline by the same route.

2.4. Sample collection and tissue homogenization

On day four post-infection (PI), all animals were anesthetized with natural anesthetic (*Cymbopogon flexuosus* essential oil) followed by spinal cord section according to the Ethics Committee recommendations. Thereafter, the kidney was removed and dissected in a glass dish over ice and divided in two parts: one part for the measurement of CK and Na^+, K^+-ATPase activities, and the other part for histopathological analyses.

In order to measure the cytosolic and mitochondrial CK activities, the renal 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-Elvehjen glass homogenizer. The homogenate was centrifuged at 10,000 × *g* for 15 min at 4 °C, and the supernatant containing cytosol and other cellular components was collected for determination of cytosolic CK activity. 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 the mitochondrial CK activity. The supernatants were stored for no more than one week at −80 °C only when the assay was not carried out immediately.

The renal tissue (100 mg) was homogenized in 1 mL of homogenization buffer (150 mM sucrose, 50 mM imidazole and 10 mM EDTA, pH 7.5) for measurement of the Na^+, K^+-ATPase activity. The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C, and the supernatant was stored at −80 °C until utilization.

2.5. Renal cytosolic and mitochondrial CK activities

Creatine kinase activity (CK) 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 of protein in a final volume of 150 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. Concentration of the 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 [19]. The color 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 absorbance was read after 20 min at 540 nm. Results were expressed as nmol of creatine formed per min per mg of protein.