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Cholinergic and adenosinergic systems exert a pro-inflammatory profile in peripheric and splenic lymphocytes of *Rhamdia quelen* experimentally infected by *Aeromonas caviae*

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ABSTRACT

It has been recognized that the cholinergic and adenosinergic systems, through the enzymes acetylcholinesterase (AChE) and adenosine deaminase (ADA), exert an important role in the immune and inflammatory responses during bacterial fish diseases. The immune response against *Aeromonas caviae* remains poorly understood, and the involvement of these systems, through the anti-inflammatory molecules acetylcholine (ACh) and adenosine (Ado), has not been studied. Thus, the aim of this study was to evaluate the involvement of cholinergic and adenosinergic systems on the inflammatory and immune responses in peripheric and splenic lymphocytes of fish experimentally infected with *A. caviae*. AChE and ADA activities in peripheric and splenic lymphocytes increased in infected animals on day 4 post-infection compared to uninfected animals, while the ACh and Ado levels decreased. Moreover, spleen histopathology revealed an increase in the number of melano-macrophage centers, as well as lipid inclusions and cellular debris in the ellipsoids. This evidence indicates that infection by *A. caviae* alters the cholinergic and adenosinergic systems, suggesting the involvement of AChE and ADA activities in the impairment of the immune system, since infection leads to a reduction in ACh and Ado levels, respectively. In summary, the upregulation of AChE and ADA activities in peripheric and splenic lymphocytes exerts a pro-inflammatory profile, contributing to systemic and tissue inflammatory damage, and consequently to disease pathogenesis.

1. Introduction

Aquaculture provides large quantities of fish in biological and economically efficient forms, as they are a source of proteins and micronutrients important for human health (Marinho et al., 2013; Reverter et al., 2014). However, infectious bacterial diseases, such as caused by *Aeromonas caviae*, cause high economic losses for fish farming, due to high mortality rates; this is considered the major impediment to the development of aquaculture (Giri et al., 2015).

Aeromonas caviae is an important pathogenic, Gram-negative bacterium that is emerging globally in a diverse range of fish species, including rainbow trout (*Oncorhynchus mykiss*) (Zepeda-Velázquez et al., 2017), tambaqui (*Colossoma macropomum*) (Marques et al., 2016), Nile tilapia (*Oreochromis niloticus*) (Peepim et al., 2016), Indian catfish (*Clarias batrachus*) (Thomas et al., 2013), rohu carp (*Labeo rohita*) (Indu and Kumar, 2012) and silver catfish (*Rhamdia quelen*) (Baldissera et al., 2017a). The most common symptoms caused by *A. caviae* include ulcerative lesions on the body surface, bases of the fins and around the anus, abdominal distension and exophthalmia, and minor symptoms of anemia, hepatomegaly and ascitis (Lowry and Smith, 2007; Thomas et al., 2013). To our knowledge, there is no data regarding the effects of this bacterium on immune system, such as the effects on enzymes belonging to the cholinergic and adenosinergic systems, which are considered novel markers of the inflammatory response (Do Carmo et al., 2015; Tonin et al., 2016).

The immune system is considered a system of biological structures and processes within an organism that detect a wide variety of infectious agents, such as bacteria (Rauta et al., 2012), and the splenic

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tissue exerts an essential role for the proper functioning of several host defense system mechanisms (Seker et al., 2011). In fish, the spleen is a secondary lymphatic and scavenging organ that plays a vital role in hematopoiesis, antigen degradation, antibody production processing and antigen trapping (Lefebvre et al., 2004). Moreover, the lymphocytes present in the melano-macrophage centers (or macrophage centers) from splenic tissue are capable of trapping antigens and presenting them to lymphocytes. Consequently antibodies are produced, which contributes to the defense against infectious agents (Zelikoff, 1998). Based on this evidence, we decided to evaluate the activities of enzymes belonging to the cholinergic and adenosinergic systems in the peripheric and splenic lymphocytes.

Several studies have reported the involvement of the cholinergic and adenosinergic systems in the inflammatory and immune responses during bacterial infections through the activities of acetylcholinesterase (AChE) and adenosine deaminase (ADA) (Da Silva et al., 2016; Boiago et al., 2016). AChE hydrolyzes acetylcholine (ACh), a neurotransmitter with an anti-inflammatory profile that can modulate the immune response, into choline and acetate, contributing to preventing inflammatory damage (Anglister et al., 2008), such as observed by Baldissera et al. (2016) in fish experimentally infected with *A. hydrophila*. The adenosinergic system is another important anti-inflammatory pathway and is composed of ADA, an important enzyme in purine metabolism that catalyzes the irreversible deamination of adenosine (Ado) into inosine (Ruiz et al., 2000). ADA activity exerts an important role on the immune and inflammatory responses due to the regulation of Ado levels, a molecule with anti-inflammatory properties involved in the differentiation and proliferation of lymphocytes (Frode and Medeiros, 2011), as observed in fish infected with *A. hydrophila* (Baldissera et al., 2016). On the other hand, the upregulation of AChE and ADA activities have been linked with pro-inflammatory profiles due to the decrease in ACh and Ado levels, respectively, contributing to inflammatory damage, as observed in the lymphocytes of fish infected with *Pseudomonas aeruginosa* (Baldissera et al., 2017b).

Based on this evidence, the aim of this study was to evaluate the involvement of cholinergic and adenosinergic systems on the inflammatory and immune responses in the peripheric and splenic lymphocytes of fish experimentally infected with *A. caviae*.

2. Materials and methods

2.1. Fish harvesting, maintenance and water quality variables

Healthy fish were collected for experimental purposes from a fish farm located in Cruz Alta, southern Brazil. The animals were transported to the Fish Physiology Laboratory at the Universidade Federal de Santa Maria, where they were maintained in 250 L fiberglass tanks with continuous aeration under controlled water variables in freshwater for 7 days: temperature 18–20 °C (maintained with air conditioner), pH 7.1–7.3 and dissolved oxygen levels 5.9–7.5 mg/L. Dissolved oxygen and temperature were measured with a YSI oxygen meter (Model 75512, 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. (1978) and non-ionized ammonia levels were measured according to Colt (2001), as recently published in detail by Baldissera et al. (2017a). 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.

2.2. Inoculum confirmation and preparation

The pathogen identity was confirmed by colony morphology and physiological characteristics, as well as the analysis of the 16S rRNA gene of *A. caviae,* as published recently in detail by Baldissera et al. (2017a).

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 experimental design

Seventy-two adult silver catfish (118 ± 21 g; 30 ± 3 cm) were used as the experimental model to assess the AChE and ADA activities and the ACh and Ado levels in peripheric and splenic lymphocytes. Fish were assigned into two groups with six animals each (six repetitions of each group): uninfected animals (negative control group) and experimentally infected animals (positive control group) were inoculated intramuscularly with 100 µL of a bacterial suspension containing 55×10^6 viable cells of *A. caviae,* according the protocol established by Baldissera et al. (2017a). The negative control group received the same dose of sterile saline by the same route.

The methodology used in the experiment was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria under protocol number 074/2014.

2.4. Sample collection

On day 4 post-infection (PI), blood samples were collected from the caudal vein using heparinized syringes for lymphocyte isolation to measure AChE and ADA activities, as well as the ACh and Ado levels. Thereafter, the spleen was removed and dissected in a glass dish over ice and divided into two parts: one for lymphocyte isolation to measure AChE and ADA activities and ACh and Ado levels, and the other for histopathological analyses. All animals were euthanized using a natural anesthetic (*Cymbopogon flexuosus* essential oil) followed by spinal cord dissection according to the Ethics Committee recommendations.

2.5. Lymphocyte isolation

Peripheral and splenic lymphocytes were isolated using a Ficoll Hypaque density gradient as described by Boyum (1968), as recent published in detail by Baldissera et al. (2017b).

2.6. Lymphocytic AChE activity

Lymphocytic AChE activity was determined according to the method described by Ellman et al. (1961) and modified by Castro et al. (2017). Briefly, proteins of all samples were adjusted to 0.1–0.2 mg/mL, and 0.2 mL of intact cells was added to a solution containing 1.0 mmol acetylcholine (ACh), 0.1 mmol DTNB and 0.1 mmol 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. AChE activity was calculated from the quotient between lymphocyte AChE activity and protein content, and the results were expressed as µmol AcSCh/h/mg of protein.

2.7. Lymphocytic ADA activity

Lymphocytic ADA activity was measured spectrophotometrically according to the method of Giusti and Gakis (1971). The reaction was started by addition of the substrate (Ado) to a final concentration of 21 mmol/L and the incubation was carried out for 60 min at 37 °C with 50 µL of peripheral or splenic lymphocytes. The reaction was stopped by adding 1.5 mL of 106/0.16 mmol/L phenol-nitroprusside to the reaction mixture, which was immediately mixed with 1.5 mL of 125/11 mmol/L of alkaline-hypochlorite (sodium hypochlorite). The specific ADA activity was expressed as U/mg of protein.

2.8. Lymphocytic ACh levels

The lymphocytic ACh levels were estimated using a commercial