Microbial Pathogenesis 122 (2018) 53–57

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

The disturbance of antioxidant/oxidant balance in fish experimentally infected by *Aeromonas caviae*: Relationship with disease pathophysiology

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ARTICLE INFO

Keywords:
Aquaculture disease
Disease pathophysiology
Oxidative damage
Reactive oxygen species

ABSTRACT

Aeromonas caviae is a Gram-negative bacterium rarely found in fish but it can be associated to high mortality of infected animals. The disease pathogenesis in fish associated to liver and kidney lesions directly linked to the initiation and progression of the disease remains poorly understood. Thus, the aim of this study was to evaluate whether *A. caviae* infection causes oxidative stress in liver and kidney of silver catfish *Rhamdia quelen,* and its involvement in disease pathogenesis. Reactive oxygen species (ROS) and thiobarbituric acid reactive substances (TBARS) levels increased in liver and kidney of fish experimentally infected by *A. caviae* compared to the control uninfected group. On the other hand, non-protein sulfhydryl (NPSH) levels decreased in both tissues of infected animals, while the glutathione S-transferase (GST) activity decreased only in the hepatic tissue. No difference was observed between groups in both tissues regarding superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) activities and glutathione (GSH) levels. In summary, the disturbance of hepatic and renal antioxidant/oxidant equilibrium contributes to the pathophysiology of the disease in fish experimentally infected by *A. caviae.*

1. Introduction

Aeromonas sp. are commonly found in aquatic environment throughout the world, and are the causative agent of significant diseases in fish such as *A. hydrophila* (agent of epizootic ulcerative syndrome) and *A. salmonicida* (agent of furunculosis) [1–3]. In addition, *Aeromonas caviae* has also gained attention as an important pathogen for fish with an incidence of 1–2% in relation to the others Aeromonas species [3]. It is a Gram-negative anaerobic bacterium found in aquatic environments, including aquacultures [1], that infects a vast number of freshwater fishes, such as African catfish (*Clarias gariepinus*) [2], rainbow trout (*Oncorhynchus mykiss*) [3], tambaqui (*Colossoma macropomum*) [4], and silver catfish (*Rhamdia quelen*) [5]. In fish, this disease is related to hepatosplenomegaly, skins lesions and impairment of the hepatic energetic metabolism, and has gained recognition in fish culture as one of the most prevalent causative agents of hemorrhagic septicemia [5,6]. Liver and kidney are the main organs affected by *A. caviae* [7], but the pathological effects of its infection in these tissues remain poorly understood. Thus, more studies are needed to understand the mechanisms involved in disease pathophysiology, such as the role of the oxidative stress, which is directly linked to the initiation and progression of infectious diseases [8].

Oxidative stress is considered as a disturbance in the antioxidants/oxidant status in favor of the former, which happens when the synthesis of free radicals is faster than their scavenge by antioxidant mechanisms [9], contributing to conformation changes in macromolecules as lipids, proteins, and nucleic acid, and consequently, tissue damages and losses of their biological functions [10]. In order to prevent or minimize the production of free radicals, such as reactive oxygen species (ROS), most organisms activate the antioxidant defense system to limit the pro-oxidant activity of ROS [11]. Recently, several evidences have demonstrated the involvement of oxidative stress in the pathophysiology

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https://doi.org/10.1016/j.micpath.2018.06.011
Received 9 April 2018; Received in revised form 3 June 2018; Accepted 6 June 2018
Available online 07 June 2018

of bacterial infectious diseases [12], including during fish bacterial infection [13]. According to these authors, the increase of lipid peroxidation and protein carbonylation levels, as well as the impairment of hepatic antioxidant status, are directly correlated to disease pathophysiology and clinical signs of silver catfish experimentally infected by *Aeromonas hydrophila* [13]. Moreover, a study conducted by Kurhalyuk and Tkachenko [14] demonstrated that *A. hydrophila* induced oxidative stress and impaired the hepatic antioxidant defense system in sea trout (*Salmo trutta* L.) naturally infected, which corroborated to the involvement of oxidative stress in the pathogenesis of ulcerative dermal necrosis as observed in fish experimentally infected by *Streptococcus agalactiae* [15].

Based on these evidences, the aim of this study was to evaluate whether *A. caviae* infection causes oxidative stress in liver and kidney of silver catfish, and its involvement in disease pathophysiology.

2. Material and methods

2.1. Fish harvesting and maintenance and water quality variables

Healthy fish were collected for experimental purposes from a fish farm located in Southern Brazil and transported to the Laboratory of Fish Physiology at the Universidade Federal de Santa Maria (UFSM), where they were maintained for seven days in 250 L fiberglass tanks containing freshwater with continuous aeration under controlled water variables: temperature 18–20 °C (maintained with air conditioner), pH 7.1–7.3 and dissolved oxygen levels of 5.9–7.5 mg/L. Dissolved oxygen, temperature, total ammonia, and non-ionized ammonia levels were determined as recently reported in detail by Baldissera et al. [5] and remained stable throughout the experimental period. 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 *A. caviae* strain was obtained from a naturally infected fish, and was confirmed by its physiological characteristics and using polymerase chain reaction (PCR) 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 details by Baldissera et al. [5]. The amplification product was subjected to direct sequencing, and a 100% match to GeneBank sequence accession number X60408.1 (http://www.ncbi.nlm.nih.gov) of *A. caviae* was noted. The specific action of the bacterial isolate isolated from naturally infected fish as a pathogen was confirmed by re-isolating the bacterium from the spleen and liver of infected fish to satisfy Koch's postulates. Finally, the gene 16S ribosomal RNA of *A. caviae* was amplified by polymerase chain reaction (PCR), while no amplifications were observed using the same set of primers with other reference bacterial species (*A. hydrophila, A. salmonicida* and *A. rivipollensis*). The bacterial isolate was grown in nutrient agar to be used 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 [5].

2.3. Fish and experimental design

Twenty adult silver catfish (*R. quelen*) (118 ± 21 g; 30 ± 3 cm) were used as the experimental model to assess parameters of oxidative stress in liver and kidney. The animals were divided into two groups with 10 animals each: uninfected animals (the negative control group) and experimentally infected animals (the positive control group) inoculated intramuscularly with 100 µL of a bacterial suspension containing 5.5×10^7 viable cells of *A. caviae,* according to the protocol established by Baldissera et al. [5], which demonstrated that *A. caviae* was able to induce hepatic alterations associated to energy metabolism. 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 and tissue homogenization

On day four post-infection (PI), all animals were anesthetized with a natural anesthetic compound (*Cymbopogon flexuosus* essential oil) followed by the spinal cord section according to the Ethics Committee recommendations. Thereafter, the liver and kidney were removed and dissected in a glass dish over ice and homogenized (1:10 w/v) in a glass Potter tube with Tris-HCl buffer (10 mM, pH 7.4), centrifuged at $2000 \times g$ for 10 min. Aliquots of each supernatant were stored at −20 °C until utilization. The protein content was tested by the Bradford method and standardized by the bovine serum albumin method [16].

2.5. Oxidative stress parameters

2.5.1. Reactive oxygen levels (ROS)

ROS levels were determined by the DCFH oxidation method described by LeBel et al. [17], recently published in details by Biazus et al. [12]. Fluorescence was measured using excitation and emission wavelengths of 485 and 538 nm, respectively. A calibration curve was established with 2′,7′-dichlorofluorescein (DCF) (0.1 nM–1 µM) as the standard, and results were expressed as U DCF/mg of protein.

2.5.2. Thiobarbituric acid reactive substances (TBARS)

As an index of lipid peroxidation, TBARS formation during an acid-heating reaction was determined as previously described by Ohkawa et al. [18]. Malondialdehyde (MDA) solution was used as a reference standard. TBARS levels were determined by the absorbance at 532 nm and results were expressed as MDA equivalent (nmol MDA/mg of protein).

2.5.3. Superoxide dismutase (SOD) activity

SOD activity was spectrophotometrically evaluated as described by Marklund and Marklund [19], and recently published in details by Souza et al. [20]. The enzymatic activity was expressed as SOD units/mg of protein.

2.5.4. Non-protein thiols (NPSH) levels

NPSH levels were determined colorimetrically at 412 nm as previously described by Ellman [21] and published in details by Souza et al. [20]. A cysteine solution was used as reference standard. NPSH were expressed as µmol SH/g of tissue.

2.5.5. Glutathione metabolism

GPx activity was measured according the methodology described by Paglia and Valentine [22], which is based on the oxidation of NAPDH at absorbance of 340 nm, as reported in details by Biazus et al. [12]. The enzymatic activity was expressed as U/mg of protein. GR activity was measured according to the methodology described by Carlberg and Mannervik [23], which is based on the utilization of the enzyme oxidized glutathione (GSSG) to convert GSSG to GSH in the presence of cofactor NADPH at absorbance of 340 nm, as reported in details by Biazus et al. [12]. The enzymatic activity was expressed as U/mg of protein. GST activity was measured spectrophotometrically at 340 nm by the method described by Habig et al. [24], as recently published in details by Biazus et al. [12]. The enzymatic activity was expressed as U/mg of protein. GSH levels were measured according to the methodology described by Browne and Armstrong [25], which is based on the reaction of GSH with the fluorophore *o*-phthaldehyde (OPT) using the sample by deproteinization with metaphosphoric acid at excitation