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Melaleuca alternifolia essential oil nanoparticles ameliorate the hepatic antioxidant/oxidant status of silver catfish experimentally infected with *Pseudomonas aeruginosa*

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ABSTRACT

Oxidative stress has been recognized as a conjoint pathological mechanism that contributes to initiation and progression of liver injury, such as that caused by bacterial diseases. Natural antioxidants are considered a rational curative strategy to prevent and cure hepatic diseases involved with oxidative stress. Thus, the aim of this study was to evaluate, for the first time, whether treatment with bactericidal *Melaleuca alternifolia* essential oil (TTO) nanoparticles prevents or reduces the hepatic damage in silver catfish (*Rhamdia quelen*) experimentally infected with *Pseudomonas aeruginosa* (PAO1). Liver samples from fish infected with *P. aeruginosa* showed increased thiobarbituric acid reactive substances (TBARS), protein carbonylation and advanced oxidation protein product (AOPP) levels, while catalase (CAT) activity was reduced compared to uninfected animals. The prophylactic treatment with nanoencapsulated TTO prevented these alterations. Based on this evidence, we concluded that *P. aeruginosa* infection causes hepatic damage, evidenced by increased TBARS, protein carbonylation and AOPP levels, which inhibits the antioxidant defense system, contributing to disease pathophysiology. Thus, this treatment may be considered an important approach for the prevention of hepatic oxidative damage caused by *P. aeruginosa* infection in fish.

1. Introduction

Fish farms produce large quantities of fish in a biologically and economically efficient way, since fish and/or fishery products represent an important source of protein and essential micronutrients for human health [1]. However, intensive fish culture enhances the probability of infectious diseases, such as that caused by *Pseudomonas aeruginosa* [2], which are recognized as the major impediment of the development of aquaculture and are often the most significant cause of economic loss because they reduce the production rate and flesh quality [3].

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen widespread in nature, inhabiting soil, water, humans, animals and fishes [4], considered to be one of the most common bacterial pathogens in marine and freshwater aquaculture, including in the silver catfish (*Rhamdia quelen*) [2]. In fish, this disease leads to development of the so-called Red Skin Disease, being characterized by petechial hemorrhage, darkness of the skin, abdominal ascitis, exophthalmia, hemorrhagic septicemia, tail and fin rot and behavioral alterations [4,5], contributing to high mortality and economic losses for fish producers [6]. It is important to emphasize that strain PAO1 can colonize a surface by forming biofilm, a mechanism of resistance linked with tissue invasion, as well as causing tissue and cellular damage, contributing to disease pathogenesis [7]. Recent studies demonstrated that bacterial diseases alter the antioxidant/oxidant status [8,9], such as that observed in the hepatic tissue of silver catfish experimentally

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infected with *Aeromonas hydrophila* [10], contributing to disease pathophysiology. Thus, our hypothesis is that *P. aeruginosa* impairs the hepatic antioxidant/oxidant status, contributing to disease pathogenesis.

Oxidative stress is considered a disturbance between antioxidant/prooxidant status in favor of excessive generation or slower removal of free radicals, which can lead to biomolecular damage to proteins, lipids and nucleic acids, with a consequent loss of their biological functions and/or homeostatic imbalance and tissue injury [11]. Oxidative tissue damage is linked to a variety of pathological diseases [12], such as those caused by bacterial diseases [10]. To our knowledge, there are no reports on the effects of infection by this bacterium on oxidative stress variables, but some studies demonstrated that virulence factors of *P. aeruginosa*, such as pyocyanin [13] and exotoxin A [14], cause oxidative stress and significant cytotoxic effects in experimentally infected rats. Thus, our hypothesis is that *P. aeruginosa* infection alters the hepatic antioxidant/prooxidant status, leading to oxidative damage and consequently contributes to disease pathogenesis.

Recently, Li et al. [15] demonstrated that antioxidative therapy using natural antioxidants, such as essential oils, represents an interesting therapeutic approach for the prevention and treatment of hepatic damage caused by oxidative stress, including during oxidative stress caused by bacterial diseases [10]. Souza et al. [2] demonstrated that nanoencapsulation of *Melaleuca alternifolia* essential oil, popularly known as tea tree oil (TTO), exerts a potent bactericidal action in fish experimentally infected with *P. aeruginosa*. Moreover, Baldissera et al. [10] demonstrated that TTO is considered an important approach to prevent the hepatic damage caused by *A. hydrophila* infection in fish. Thus, our hypothesis is that *M. alternifolia* essential oil nanoparticles may reduce or prevent the hepatic damage caused by *P. aeruginosa*.

Based on this evidence, the aim of this study was to evaluate whether TTO nanoparticles are capable of preventing or reducing hepatic damage in silver catfish experimentally infected with *P. aeruginosa*.

2. Materials and methods

2.1. TTO and nanoencapsulated TTO

The TTO was obtained from Delaware (Brazil) and the nanocapsules containing TTO were obtained from Inventiva (Porto Alegre, Brazil). TTO composition was analyzed by gas chromatography (GC) using an Agilent Technologies 6890 N GC-FID system, equipped with a DB-5 capillary column connected to a flame ionization detector, as recently published in detail by Grando et al. [16], and the identification of TTO components was based on the retention index and mass spectra literature data [17]. A total of 15 compounds representing 95.86% of the total composition were identified, terpinen-4-ol (41.98%) being the principal compound, as published in detail by Grando et al. [16].

Nanostructured lipid carriers were prepared with 7.5% (w/v) TTO, based on a high pressure homogenization method, as recently published in detail by Comin et al. [18].

2.2. Physical-chemical properties of nanostructured TTO

The determination of diameter (nm), polydispersion index, zeta potential (mV) and hydrogen potential (pH) were recently described in detail by Comin et al. [18]. The nanoparticles presented a diameter of 150.2 ± 2 nm, polydispersion index of 0.213 ± 0.017, zeta potential of −8.69 ± 0.80 mV and pH of 6.3 ± 0.3.

2.3. Collection and maintenance of fish and water quality parameters

Healthy fish were collected for experimental purposes from a fish farm located in Rio Grande do Sul state, Brazil. The fish were transported alive and maintained in 250 L fiberglass tanks with continuous aeration and controlled water parameters (21–23 °C, pH 7.2–7.6, dissolved oxygen levels: 5.6–7.2 mg/L) in fresh water for 14 days. Dissolved oxygen and temperature were measured with a YSI oxygen meter (Model Y5512, Ohio, USA). The pH was measured using a DMPH-2 pH meter (Digimed, São Paulo, Brazil). Total ammonia levels were determined according to Verdouw et al. [19] and non-ionized ammonia (NH_3) levels were calculated according to Colt [20], recently published in detail by Baldissera et al. [5]. The animals were fed to apparent satiation with commercial feed once a day. Any uneaten food, feces and other residues were removed daily 30 min after feeding.

2.4. Bacterial culture and inoculum preparation

The *P. aeruginosa* strain PAO1 was grown on MacConkey agar and nutrient agar, and turbidity (OD_{600}) adjusted to 0.8–1.0 mL (equivalent to 10^8 CFU/mL) was used for the infection model, as recently published in detail by Baldissera et al. [5].

2.5. Animal model and study design

Twenty juvenile silver catfish (70.2 ± 6.3 g; 24 ± 3 cm) were used as the experimental model to assess hepatic oxidative stress variables. Fish were assigned into four groups (A–D) with five animals each, where groups A and B were composed of uninfected animals, and groups C and D were composed of infected animals. Groups A and C were used as the negative control and positive control (without treatment), respectively. Animals from groups B and D were exposed to prophylactic treatment for 7 days, which consisted of 1 h of exposure per day at 50 μL/L of TTO nanoparticles. After this treatment, groups C and D were inoculated intramuscularly with 100 μL of *P. aeruginosa* strain PAO1 (10^8 CFU/mL) in the right laterodorsal side of each fish, according the protocol established by Souza et al. [2].

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.

2.6. Sample collection

On day 7 post-infection (PI), all animals were subsequently euthanized by sectioning the spinal cord according to the Ethics Committee recommendations. Thereafter, the liver was removed and dissected on a glass dish over ice for the measurement of oxidative stress variables. Briefly, the liver was homogenized (1: 10 w/v) in a glass Potter tube with Tris-HCl buffer (10 mM, pH 7.4), and centrifuged at 2000 × *g* for 10 min. Aliquots of the supernatant were stored at −20 °C until utilization. The protein content was tested by the Bradford method and standardized to bovine serum albumin [21].

2.7. Hepatic oxidative stress variables

2.7.1. Non-enzymatic variables

2.7.1.1. 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. [22]. A malondialdehyde (MDA) solution was used as a reference standard. TBARS levels were determined by the absorbance at