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In vitro and *in vivo* protective effect of arginine against lipopolysaccharide induced inflammatory response in the intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian)

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ABSTRACT

The present study was designed to assess the possible protective effects of arginine (Arg) against lipopolysaccharide (LPS) induced inflammatory response in juvenile Jian carp (*Cyprinus carpio* var. Jian) *in vivo* and in enterocytes *in vitro*. Firstly, inflammatory response was established by exposing enterocytes to different concentrations of LPS for 24 h. Secondly, the protective effects of Arg against subsequent LPS exposure were studied in enterocytes. Finally, we investigated whether dietary Arg supplementation could attenuate immune challenge induced by LPS *in vivo*. The result indicated that 10 mg/L LPS could induced inflammatory response in enterocytes. Cells exposed to LPS (10–30 mg/L) alone for 24 h resulted in a significant increase in lactate dehydrogenase release (LDH) ($P < 0.05$). The cell viability, protein content, alkaline phosphatase activity were decreased by LPS ($P < 0.05$). Moreover, LPS exposure significantly increased TNF-α, IL-1β, and IL-6 mRNA expression *in vitro* ($P < 0.05$). However, pre-treatment with Arg remarkably prevented the increase of TNF-α, IL-1β, and IL-6 by inhibiting the excessive activation of TLR4-Myd88 signaling pathway through down-regulating TLR4, Myd88, NFκB p65, and MAPK p38 mRNA expression ($P < 0.05$). Interestingly, the experiment *in vivo* showed that Arg pre-supplementation could attenuate immune challenge induced by LPS via TLR4-Myd88 signaling pathway, and thus protect fish against LPS-induced inflammatory response. In conclusion, all of these results indicated pre-supplementation with Arg decreased LPS induced immune damage and regulated TLR4-Myd88 signaling pathway in juvenile Jian carp *in vivo* and in enterocytes *in vitro*.

1. Introduction

Arginine (Arg) is one of the essential amino acids for fishes. Sufficient Arg in the diets is indispensable to optimize the health status of fishes [1,2]. Fish growth is often related to the disease resistance [3], which could be reflected by survival [4]. Studies showed that dietary Arg deficiency reduced the survival of coho salmon (*Oncorhynchus kisutch*) [5] and European sea bass (*Cirrhinus mrigala*) [6]. Fish disease resistance is associated with immune defense system, which includes innate and adaptive immunity. Buentello et al. demonstrated that dietary Arg supplementation enhanced the ability of channel catfish to survive exposure to *Edwardsiella Ictaluri* [2]. Pohlenz et al. reported Arg supplementation to culture media improves channel catfish macrophage phagocytosis and killing ability [7]. In mammals, dietary Arg supplementation was shown to alleviate a variety of infections and immune challenges [8,9,10]. These data suggested that Arg had a relationship with immunity. However, the potential mechanism has not been elucidated. Arg modulates immune functions through regulating several cytokines, such as interleukin-1β (IL-1β), IL-2, and tumor necrosis factor-α (TNF-α) in piglet [10]. However, the structure and form of the immune system is different between fish

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and mammals [11]. Whether Arg could improve disease resistance via regulating the cytokines in fish is unclear.

Toll-like receptor 4 (TLR4) is an important member of pattern recognition receptors (PRRs) in the immune system by recognition of pathogen-associated molecular patterns (PAMPs), which are conserved molecules commonly shared by classes of pathogens [12,13]. Upon recognition, TLR4 transmits signals to nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPK) through two signaling pathways. Namely, the myeloid differentiation primary response gene 88 (Myd88)-dependent pathway and the TIR-containing adapter inducing IFN-β (TRIF)-dependent pathway lead to changes in transcription of various effector proteins and inflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-12 [14]. Su et al. reported TLR4 signaling pathway could be activated by both viral and bacterial infection in rare minnow *Gobiocypris rarus* [15]. In Atlantic salmon *Salmo salar*, bacterial lipopolysaccharide (LPS) induced significant up-regulation of IL-1β and IL-8 mRNA expression in head kidney leucocytes [16]. Recently, LPS has been used in studies of various aspects of induced immune responses in fish enterocytes [17,18]. Fish enterocytes are an important trigger of the intestinal immune system [18]. Mulder et al. reported that *in vivo* immersion exposure of the rainbow trout to *Aeromonas salmonicida* induces the expression of several cytokines in the intestine [19]. Komatsu et al. also reported that bacterial infection resulted in inflammatory responses in the intestine of trout *Oncorhynchus mykiss* [18]. These studies focused on bacteria induced inflammatory responses in the intestine of fish. However, to our knowledge, few studies examined how to efficiently protect the intestine against bacteria-induced immune damage.

In the present experiment, we first investigated the effects of Arg on LPS-induced inflammatory responses *in vivo* and *in vitro*. And based on these effects, the potential mechanisms behind the Arg-regulated immune responses were investigated.

2. Materials and methods

2.1. Chemicals

LPS, Arg, insulin, collagenase, dispase, transferrin, benzyl penicillin, and streptomycin sulfate were purchased from Sigma (St. Louis, MO, USA). Hank's balanced salt solution (HBSS) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). Arg-free DMEM was ordered from Beijing Tsing Skywing Bio. Tech. Co. Ltd. (Beijing, China). 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) were purchased from Promega Corporation (Madison, WI).

2.2. In vitro experiments

2.2.1. Primary enterocyte culture

Cell isolation and culture were performed according to the methods of Jiang et al. (2009) [20] and Jiang et al. (2013) [21] with minor modifications. In brief, healthy carp with an average weight 48.5 ± 1.2 g were maintained for approximately 24 h without feeding before the experiment, and killed by decapitation. The intestines were rapidly removed from the carcass, opened and rinsed with HBSS containing antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Enterocytes were isolated by collagenase and dispase digestion. Cells were pooled and suspended in DMEM, and washed five times with DMEM to remove enzymes. Isolated enterocytes were seeded in 24-well culture plates (Falcon, Franklin Lake, NJ, USA) at the density of 2×10^3 cells/well that had been previously coated with collagen I (Sigma, St. Louis, MO, USA), as previously described by us [22]. The cells were cultured in DMEM supplemented with 5% FBS, 0.02 mg transferrin/mL, 0.01 mg insulin/mL, and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 26 ± 0.5 °C under a Biochemical Incubator (Shanghai Boxun Industry & Commerce Co., Ltd., Shanghai, China). The cells were allowed to attach to plates for 72 h.

2.2.2. LPS induced inflammatory response in carp enterocytes

The cells were incubated for 24 h in fresh medium containing 0, 5, 10, 20 and 30 mg/L LPS. At the end of the exposure, the MTS assay was performed. The cell lysates were collected to detect alkaline phosphatase (AKP) activity, protein content (PC), TNF-α, and IL-1β mRNA expression, the medium was collected to detect the content of lactate dehydrogenase (LDH).

2.2.3. Protective effect of Arg in LPS-induced inflammatory response in carp enterocytes

To investigate the potential protective effect of Arg against a subsequent LPS exposure, enterocytes were pretreated with 0 (negative Ctrl group, group 1), 0 (group 2), 50 (group 3), 100 (group 4), 150 (group 5), 200 (group 6), 250 (group 7), 300 (group 8) mg/L of Arg for 72 h prior to 24 h treatment with 10 mg/L LPS in a 27 °C incubator. The LPS exposure concentration was chosen because previous experiment showed that 10 mg/L LPS of medium could induce inflammatory response in carp enterocytes. Group 1 cells were incubated in Arg-free DMEM. Thus, there were seven groups (pre-treatment + LPS exposure): Ctrl + Ctrl, Ctrl + LPS, 50 mg/L Arg + LPS, 100 mg/L Arg + LPS, 150 mg/L Arg + LPS, 200 mg/L Arg + LPS, 250 mg/L Arg + LPS, 300 mg/L Arg + LPS. At the end of the experiment, media were collected to analyze LDH release. Cell lysates were collected to detect TLR4, Myd88, NF-κBp65, MAPKp38, TNF-α, IL-1β, IL-6, and IL-10 mRNA expression.

2.3. In vivo experiments

2.3.1. Animal collection and acclimation conditions

Juvenile Jian carp were obtained from Tong Wei fisheries (Sichuan, China) and acclimated for 4 weeks. The laboratory conditions were as follows: 24 ± 1 °C, constant aeration, daily dechlorinated water change, natural photoperiod and feeding with a commercial food.

2.3.2. Protective effect of Arg in LPS-induced inflammatory response in carp

The formulation of the basal diet was the same as in our previous study [1]. Briefly, it contained 340 g crude protein/kg diet. The basal diet was Arg unsupplemented control (Ctrl). Arg premix was added to the basal diet to provide 18.5 g Arg/kg diet, which was the required Arg concentration for optimal growth established by our previous study, and the amount of cellulose was reduced to compensate (Arg group). Procedures for diet preparation and storage were the same as those described by Ref. [23]. A total of 300 fish with an average initial weight of (10.53 ± 0.03 g) from the acclimatization aquarium were randomly assigned into 2 groups of 3 replicates each. The groups were fed either the Ctrl diet or the Arg diet for 63 days. The experimental conditions were the same as in our previous study [1].

At the end of the feeding trial, the fish in each aquarium were weighed and collected for LPS exposure. Fish from both the Ctrl and Arg groups were injected intraperitoneally with 100 μL of *Escherichia coli* LPS serotype 0111:B4 (3 mg of LPS kg^{-1} of fish) diluted in sterile PBS for 48 h. The Ctrl/Ctrl treatment (fish from the Ctrl) was performed by injecting with 100 μL sterile PBS. Hence, there were 3 different pre-treatment/exposure groups, Ctrl/Ctrl, Ctrl/LPS and Arg/LPS, with 3 replicates per group and 12 fish per replicate (36