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Effects of repeated handling and air exposure on the immune response and the disease resistance of gibel carp (*Carassius auratus gibelio*) over winter

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ABSTRACT

High mortalities and suppressed immune functions of farmed fish over winter are the universal problems in aquaculture. It is necessary to improve the immune response and disease resistance in the overwintering fish. A recent study suggested that repeated handling increased innate immune mechanisms and disease resistance in Senegalese sole. Therefore, the present study evaluated the hypothesis that appropriate repeated handling could compromise the immune depression and increase the disease resistance in gibel carp over winter. The experiment was executed in field net cages (2 m × 2 m × 2 m) from Dec. 4, 2012 to Apr. 2, 2013. Three cages with 50 fish per cage were randomly designed as the control group and did not receive any interfere over winter. The other three cages received repeated handling with an air exposure for 5 min every week during the experiment. Fish were not fed over winter. At the end of the trial, fish were challenged with *Aeromonas hydrophila* at a dose of 1.5×10^8 CFU ml^{-1}. The results showed that no significant difference of final body weight was found between groups. The spleen and kidney somatic index increased in the control fish after bacterial challenge and showed a rising trend but not a statistical change in repeated handled fish. Plasma cortisol levels significantly increased in the control fish at 6 h post bacterial challenge and then declined. However, repeated handled fish did not show any significant change in plasma cortisol levels after challenge. The reduced inducement of heat shock protein 70 (HSP70) expressions by repeated handling was found in gibel carp post bacterial challenge. After overwintering, the repeated handled fish exhibited increased catalase (CAT) and superoxide dismutase (SOD) activities. Enhanced plasma CAT activities and reduced plasma malondialdehyde (MDA) contents were found in repeated handled fish over time against invading bacteria. Up-regulation of myeloid differentiation primary response gene 88 (MyD88) and interleukin 11 (IL11) was observed in repeated handled fish over time after bacterial challenge. The overexpression of IL11 was significantly reduced by repeated handling against invading bacteria compared to the control group. The present results implied that a MyD88-dependent signaling pathway was involved in the innate immune responses of gibel carp by repeated handling over winter against invading bacteria.

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1. Introduction

Gibel carp (*Carassius auratus gibelio*) has become one main culture fish in China, of which the annual production is more than 2.5 million tones. Like other warm water fish species, the farmed gibel carp met the problems of the immune suppression in winter. In aquaculture, overwintering is a special stage. Fish suffer from the multiple stressors as the low water temperature, photoperiod and food deprivation. In winter, the immune function of fish is inhibited and fish is prone to be attacked by virus or bacteria [1]. In North China, overwintering leads to the mortality rate of more than 20% of fish [2]. It has been reported that the cultured gilthead sea bream (*Sparus aurata*) was affected by the winter syndrome, which causes chronic mortalities during the winter in the Mediterranean Sea [3]. In winter, cold-water vibriosis, a bacterial disease, is frequently observed in many cultured salmonids [4]. In Indian major carp (*Labeo rohita*) some non-specific immune parameters as serum lysozyme and myeloperoxidase activities were lower in winter as compared to any other season of the year [5]. Serum lysozyme activity, levels of respiratory burst by head kidney macrophages, blood cell counts and acquired antibody titres against Vibrio anguillarum, were all found to be positively correlated with season [6]. Therefore, the immune response of fish is known to vary seasonally and the immune parameters are suppressed during winter [1]. It is necessary to alleviate the immune suppression and improve the survival of the farmed fish during overwintering.

It has been reported that repeated handled fish presented an increased disease resistance than unstressed fish regardless of dietary treatment [7]. Following 14 days of daily air exposing for 3 min, they found that daily repeated handling induced a higher disease resistance in handled fish than in undisturbed fish, together with increased cellular (NO production) and humoral immune responses (plasma lysozyme and ACP activities) as well as gLYS and HIF-1 expression values at the time of bacterial infection. It was also reported that the sustained exercise might simulate the immune status of fish [8]. However, no literature about the effects of repeated handling on disease resistance and the immune response in fish during overwintering have been published. The hypothesis of the present study is that repeated handling can alleviate the immune suppression of the farmed fish in winter.

The innate immune is an indispensable form of defense against invading pathogens in vertebrates [9], and this immune system relies on the presence of pattern recognition receptors (PRRs) to recognize the pathogen-associated molecular patterns (PAMPs) for discriminating and eliminating pathogens [10,11]. Toll-like receptors (TLRs) are known as the well-characterized PRRs by differential regulation of pro-inflammatory, anti-inflammatory cytokines and co-stimulatory molecules [12]. Among TLRs, TLR2, TLR3 and TLR4 are the most thoroughly characterized family members [13]. Myeloid differentiation factor 88 (MyD88) is an important adaptor protein in the TLR signaling pathways [14]. All TLRs except TLR3 and TLR4 have been shown to require MyD88 for effective downstream signaling, dividing the TLR family between MyD88-dependent and MyD88-independent members [15]. In the case of pathogenic invading, MyD88 was up-regulated to enhance the activation of downstream transcription factors, such as NF-κB and AP-1 [16]. In the MyD88-independent TLR signaling pathway, TLR3 induce the activation of the IFN-β promoter, a function unimpaired by the knockdown of MyD88 [17].

The objective of the present study is to investigate whether the weekly air exposing for 5 min could compromise the immune suppression of gibel carp during overwintering and increase the resistance of the fish after challenged with *Aeromonas hydrophila*. In the present study, stress responses and the antioxidant functions were determined in the overwintering carp against the invading bacteria, as well as the transcriptional levels of TLR3, MyD88 and anti-inflammatory cytokine IL11.

2. Materials and methods

2.1. Experimental procedures

In November 2012, gibel carp were reared in net cages (2 m × 2 m × 2 m) in the Yangtze River at the National Seed Stock Farm of Four Major Carps (29°49′N, 112°28′E, Shishou, Hubei, China) for 4 weeks before overwintering. During the acclimation, fish were fed to satiation at 09:00 and 15:00 with a commercial feed (Catalogue No. 108, Tongwei Group Co., Ltd., Chengdu, Sichuan, China).

Fish were starved for one day prior to the experiment. The fish (316.5 ± 2.2 g) were bulk-weighed and randomly transferred into each cage (2 m × 2 m × 2 m). 3 cages of fish (n = 50 per cage) were used as the control fish without any disturbance. During the winter, other 3 cages of fish (n = 50 per cage) were weekly (at 08:00 of every Monday) pulled out of water and then the net cages were exposed in the air for 5 min. The experiment lasted from Dec. 4, 2012 to Apr. 2, 2013.

During the experiment, fish were not fed over winter. Daily water temperature was monitored and ranged from 3.5 °C to 16.0 °C (Fig. 1). The air temperature of every Monday during the experiment was presented in Table 2. Natural photoperiod was used. At the end of the experiment, fish were bulk weighed. After the fish were anesthetized with MS222 (80 mg L^{-1} tricaine methane sulfonate, Argent Chemical Laboratories Inc., Redmond, WA, USA) and weighed individually, the blood samples were obtained from the caudal vessels by heparinized syringes and centrifuged at 1000 × g (4 °C) for 15 min to get the plasma samples. PCR samples of kidney, spleen and head kidney tissues of 2 fish per cage were quickly taken, frozen in liquid nitrogen and stored at −80 °C. Then the tissues of spleen and kidney from another 2 fish per cage were weighed for calculation of tissue somatic indices. All the remaining fish were used for the following bacterial challenge experiment.

2.2. Bacterial challenge

A single colony of *A. hydrophila* grown on brain heart infusion (BHI) agar plate was isolated and cultured in BHI broth at 30 °C for 6 h and centrifuged at 3500 g for 10 min to harvest the bacteria. The pellet was suspended in PBS (pH7.2–7.4), adjusted to 1.5×10^8 CFU ml^{-1}, and used for the following experiments. In the pretest of bacterial challenge, four doses of 6×10^9 CFU ml^{-1}, 6×10^8 CFU ml^{-1}, 3×10^8 CFU ml^{-1} and 6×10^7 CFU ml^{-1} were tested. The 7-day mortalities of the fish with the same conditions to the present study challenged with the four doses were 100%, 100%, 80% and 0% respectively. Therefore, the dose of 1.5×10^8 CFU ml^{-1} were adopted in the present study.

3 cages of carp (n = 40 per cage) were injected intraperitoneally with *A. hydrophila* at a dose of 0.75×10^8 cells suspended in 500 μl PBS per fish. Triplicates of 40 fish per cage were intraperitoneally injected with 500 μl sterile PBS per fish as the control group. Injections were carried out under anesthesia with MS222 at 80 mg L^{-1}. Average weight of the gibel carp was 302.7 g.

At 6 h, 24 h, 72 h, 168 h after bacterial challenge, 6 fish per group were anesthetized with MS222 and weighed individually. The blood samples were obtained from the caudal vessels by heparinized syringes and centrifuged at 1000 × g (4 °C) for 15 min to get the plasma samples. PCR samples of kidney, spleen and head

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