Fish & Shellfish Immunology 46 (2015) 406–416

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Manganese deficiency or excess caused the depression of intestinal immunity, induction of inflammation and dysfunction of the intestinal physical barrier, as regulated by NF-κB, TOR and Nrf2 signalling, in grass carp (*Ctenopharyngodon idella*)

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ARTICLE INFO

Article history:
Received 17 February 2015
Received in revised form
3 June 2015
Accepted 7 June 2015
Available online 10 June 2015

Keywords:
Manganese
Cytokine
Tight junction
Antioxidant status
TOR
Nrf2

ABSTRACT

Intestinal mucosal immune components and mRNA levels of inflammatory cytokines, tight junction proteins, antioxidant enzymes and related signalling molecules in young grass carp (*Ctenopharyngodon idellus*) under dietary manganese (Mn) deficiency or excess were investigated. Fish were fed the diets containing graded levels of Mn [3.65–27.86 mg Mn kg^{-1} diet] for 8 weeks. The results demonstrated that Mn deficiency significantly decreased the lysozyme and acid phosphatase (ACP) activities, up-regulated tumour necrosis factor α (TNF-α), interleukin 8 and the signalling factor nuclear factor-κB p65, and down-regulated interleukin 10 (IL-10), transforming growth factor β1, inhibitor of signalling factors κB-α and target of rapamycin mRNA levels in the proximal intestine (PI), mid intestine (MI) and distal intestine (DI). However, Mn deficiency did not change the C3 content in the PI, whereas it decreased the C3 contents in the MI and DI. Additionally, Mn depletion also resulted in significantly low mRNA levels for tight junction proteins (claudin-b, claudin-c, claudin-15, occludin and zonula occludens-1), antioxidant enzymes (MnSOD, GPx and CAT) and NF-E2-related factor-2 in the intestines of fish. Excessive Mn exhibited toxic effects similar to Mn deficiency, where optimal Mn contents reversed those indicators. In conclusion, Mn deficiency or excess causes the depression of intestinal immunity, induction of inflammation and dysfunction of the intestinal physical barrier relating to NF-κB, TOR and Nrf2 signalling in grass carp. Furthermore, quadratic regression analysis at 95% maximum response of lysozyme and acid phosphatase activities in the distal intestine of young grass carp revealed the optimum dietary Mn levels to be 8.90 and 8.99 mg kg^{-1} diet, respectively.

1. Introduction

The intestine is constantly exposed to a wide variety of foreign substances, such as microorganisms, viruses and antigens, derived from the ingested food [1,2]. Damage to the intestinal immune system impairs the immune response, leading to bacterial translocation and enteritis and even resulting in high mortality in fish [3]. Hence, maintaining the intestinal immune function is of utmost importance to the health of fish. Intestinal immunity appears to be closely related to the composition of dietary nutrients in fish. Manganese (Mn) is known to be an essential trace element for fish [4]. Our previous study demonstrated that dietary Mn deficiency or

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http://dx.doi.org/10.1016/j.fsi.2015.06.007
1050-4648/

excess caused intestinal oxidative damage and thus reduced the digestive and absorptive abilities, as well as poor growth performance in young grass carp (*Ctenopharyngodon idellus*), whereas optimal Mn supplementation could reverse those negative effects [5]. The digestion and absorption ability of the intestine depends on the intestinal health status, which is associated with intestinal mucosal immune and barrier function [6]. However, studies to investigate the effects of Mn on intestinal mucosal immune and barrier function in fish have not been conducted, although this subject is worthy of investigation.

In fish, the intestinal immune barrier primarily depends on gut-associated lymphoid tissue, which consists of variably sized immune cells, such as lymphocytes, monocytes and macrophages [7]. These immune cells, which produce immune molecules, such as lysozyme, acid phosphatase (ACP) and complement, act as an important line of defence against attachment and penetration by potentially harmful agents in fish, such as olive flounder (*Paralichthys olivaceus*) [8] and grass carp [6]. However, few studies have been conducted to investigate the effects of Mn on intestinal mucosal components in fish. In mice, Mn could increase the macrophage activity [9]. One study showed that macrophages could secrete the lysozyme in fish [10]. Accordingly, these lines of evidence indicated that Mn might influence the intestinal mucosal components in animals, thereby warranting investigation in this area.

The intestinal immune response is often accompanied by inflammation, which is primarily mediated by cytokines [11]. To the best of our knowledge, the cytokines are regulated by signalling molecules, such as nuclear factor-κB (NF-κB) [12] and target of rapamycin (TOR) [13] in terrestrial animals. However, there are no reports concerning the effects of Mn on NF-κB- and TOR-regulated cytokine production in aquatic animals. In human peripheral blood mononuclear cells (PBMCs), the activation of NF-κB could be suppressed by dopamine [14]. One study has shown that Mn could increase the content of dopamine in neonatal rat brain [15]. In addition, Mn deficiency down-regulated the protein expression of glutamine synthetase in rat cortical astrocytes [16], which could catalyze the synthesis of glutamine in rat glial cells [17]. It has been indicated that glutamine is essential to sustaining TORC1 activity in yeast [18]. Based on these studies, it can be inferred that Mn might affect the cytokines by influencing the signalling molecules NF-κB and TOR in fish, which warrants further investigation.

Intestinal immunity has been associated with intestinal barrier function in humans [19]. As an important part of the intestinal barrier, the intestinal physical barrier consists of tight junction proteins (TJs), such as claudin, occludin and zonula occludens (ZO), in human colon adenocarcinoma (Caco-2) cells [20]. However, there is little information concerning the effects of dietary Mn on tight junction proteins in animals. It has been shown that TJs could be damaged by oxidative injury in human colonic Caco-2 cells [21]. Our previous study showed that Mn deficiency or excess caused oxidative damage and was associated with the decreases in MnSOD, GPx and CAT activities in the intestines of young grass carp [5]. Those observations indicated potential effects of Mn deficiency or excess on the TJs, which was related to the redox balance in the intestines of fish. To the best of our knowledge, the activities of enzymes might be partly related to their gene expression levels [22], which are regulated by the key transcription factor, NF-E2-related factor-2 (Nrf2) in fish [23]. To date, there is no information concerning whether Mn regulated gene expression levels of antioxidant enzymes through the Nrf2 signalling pathway in fish. It has been shown that Mn deficiency in growing rat brain led to the decrease of catecholamine synthesis [15]. Epinephrine, an important member of the catecholamine family of neurotransmitters [24], could induce the Nrf2 activity in rat astrocytes [25]. These lines of evidence indicated that Mn might affect the signalling molecule Nrf2 to influence the antioxidant enzymes and thus modulate the tight junctions in the intestines of animals, a possibility that warrants investigation.

The present study is a part of a larger study and used the same growth trial as our previous study, which reported that Mn deficiency or excess caused growth reduction and impaired the digestive and absorptive capacity of young grass carp and that the optimal Mn requirement for growth is evaluated to be 16.91 mg Mn kg^{-1} diet [5]. The objectives of the present study were intended to further investigate the effects of Mn on the intestinal mucosal immune components, cytokines, tight junction proteins and antioxidant enzymes, as well as the related signalling factors, NF-κB, TOR and Nrf2, in grass carp. The finding of this study might provide a theoretical basis to reveal the potential regulatory approach for intestinal health influenced by Mn in fish. Additionally, the dietary optimal Mn levels based on the intestinal immune-related parameters for young grass carp were also estimated.

2. Materials and methods

2.1. Experimental diets and fish trial

The formulation of the basal diet was the same as our previous study [5] and is presented in Table 1. Fish meal, casein and gelatin were used as dietary protein sources to prepare a low Mn diet. Our previous study observed that the optimal Mn requirement for growth is 16.91 mg Mn kg^{-1} diet [5]. Thus, in this study, Mn sulphate monohydrate ($MnSO_4 \cdot H_2O$) was added to the basal diet to provide graded concentrations of 0 (basal diet), 5, 10, 15, 20 and 25 mg Mn kg^{-1} diet at the expense of a corresponding amount of cellulose according to the method described by Tan et al. [26]. The Mn concentrations in the experimental diets were determined to be 3.65 (un-supplemented control), 8.62, 13.48, 18.24, 22.97 and 27.86 mg Mn kg^{-1} diet analyzed by flame atomic absorption spectrometry (novAA400, Analyticjena AG, Germany) according to the method described by Tan et al. [26]. The diet preparation procedures and storage (−20 °C) were the same as in our previous study [5].

The experimental conditions and fish feeding were all the same as our previous study [5]. Young grass carp were obtained from local fisheries (Sichuan, China) and acclimated to the experimental conditions for 2 weeks. During the acclimatization period, the fish were fed the basal diet to decrease the body Mn concentration according to the method described by Lin et al. [27]. A total of 540 fish (average initial weight 264 ± 1 g) were randomly distributed into 6 groups of 3 replicates each after the acclimatization period. The fish in each treatment cage were fed the corresponding diet four times a day to apparent satiety for 8 weeks according to our previous study [5].

2.2. Sample collection and analysis

After the feeding trial, fish were starved for 12 h according to our previous study [28]. Eighteen fish from each group were randomly selected, and then anaesthetized in a benzocaine bath (50 mg L^{-1}) according to the method described by Berdikova Bohne et al. [29]. After sacrifice, the intestines of fish were quickly removed on ice, rinsed in cold phosphate buffered saline (PBS), frozen in liquid nitrogen and then stored at −80 °C until analyzed according to the method described by Mulder et al. [30] and in our previous study [28]. The intestine samples were homogenized on ice in 10 volumes (w v^{-1}) of ice-cold physiological saline and centrifugated at 6000 *g* for 20 min at 4 °C, and the supernatants were stored as described by Deng et al. [31] until used for the determination of intestinal