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Dietary vitamin C deficiency depressed the gill physical barriers and immune barriers referring to Nrf2, apoptosis, MLCK, NF-κB and TOR signaling in grass carp (*Ctenopharyngodon idella*) under infection of *Flavobacterium columnare*

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ABSTRACT

This study explored the effects of vitamin C on the physical barriers and immune barriers, and relative mRNA levels of signaling molecules in the gill of grass carp (*Ctenopharyngodon idella*) under infection of *Flavobacterium columnare*. The results indicated that compared with optimal vitamin C supplementation, vitamin C deficiency (2.9 mg/kg diet) (1) increased reactive oxygen species, malondialdehyde and protein carbonyl (PC) contents ($P < 0.05$), decreased the copper/zinc superoxide dismutase, manganese superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activities and mRNA levels ($P < 0.05$), and glutathione and vitamin C contents ($P < 0.05$), down-regulated NF-E2-related factor 2 mRNA level ($P < 0.05$), and up-regulated Kelch-like ECH-associating protein (Keap) 1a (rather than Keap1b) mRNA level ($P < 0.05$) in the gill of grass carp under infection of *F. columnare*, suggesting that vitamin C deficiency induced oxidative injury in fish gill; (2) up-regulated caspase-3, -7, -8, -9, Fas ligand, B-cell lymphoma protein 2 associated X protein, apoptotic protease activating factor-1 mRNA levels ($P < 0.05$), and down-regulated inhibitor of apoptosis protein and B-cell lymphoma-2 (rather than myeloid cell leukemia-1) mRNA level ($P < 0.05$) in the gill of grass carp under infection of *F. columnare*, suggesting that vitamin C deficiency aggravated cell apoptosis in fish gill; (3) up-regulated pore-forming TJs Claudin-12, 15a, -15b, and related signaling molecules myosin light chain kinase, p38 mitogen-activated protein kinase (rather than c-Jun N-terminal kinases) mRNA levels ($P < 0.05$), and down-regulated barrier-forming TJs Occludin, zonula occludens (ZO) 1, ZO-2, Claudin-c, -3c, -7a, -7b mRNA levels ($P < 0.05$) in the gill of grass carp under infection of *F. columnare*, suggesting that vitamin C deficiency disrupted tight junctional complexes in fish gill; (4) decreased lysozyme and acid phosphatase (ACP) activities, and complement 3 (C3), C4 and IgM contents ($P < 0.05$), down-regulated the mRNA levels of antimicrobial peptides liver expressed antimicrobial peptide (LEAP) 2A, LEAP-2B, Hepcidin, β-defensin mRNA levels ($P < 0.05$) in the gill of grass carp under infection of *F. columnare*, suggesting that vitamin C deficiency decrease fish gill immune function; (5) down-regulated the mRNA levels of anti-inflammatory cytokines-related factors interleukin 10 (IL-10), IL-11, transforming growth factor (TGF) β1, TGF-β2, inhibitor of κBa and eIF4E-binding protein 1 (4E-BP1) (rather than 4E-BP2) ($P < 0.05$), and up-regulated pro-inflammatory cytokines-related factors interferon γ2, IL-1β, IL-6, IL-8, IL-12 P35, IL-12 P40, nuclear factor κB (NF-κB) p65 (rather than NF-κB p52), IκB kinases (IKK) (only IKKα and IKKγ), target of rapamycin and ribosomal protein S6 kinase 1 mRNA levels ($P < 0.05$) in the gill of grass carp under infection of *F. columnare*, suggesting that vitamin C deficiency aggravated fish gill inflammation. In

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conclusion, vitamin C deficiency disrupted physical barriers and immune barriers, and regulated relative mRNA levels of signaling molecules in fish gill. The vitamin C requirement for against gill rot morbidity of grass carp (264–1031 g) was estimated to be 156.0 mg/kg diet. In addition, based on the gill biochemical indices (antioxidant indices MDA, PC and vitamin C contents, and immune indices LA and ACP activity) the vitamin C requirements for grass carp (264–1031 g) were estimated to be 116.8, 156.6, 110.8, 57.8 and 134.9 mg/kg diet, respectively.

1. Introduction

Gill, as the first-line encounter towards pathogens, plays an important role in fish immune response [1]. Fish gill have developed physical barriers and immune barriers to prevent foreign pathogens invasion [2]. It was reported that impaired gill physical barriers and immune barriers result in a poor growth performance [3] and even high mortality [4] in fish. Up to now, limited studies have shown that nutrients could enhance gill physical and immune barrier functions, such as arginine and valine [5,6]. Vitamin C is an essential water-soluble vitamin for fish [7]. Our previous studies observed that optimal vitamin C could increase the growth performance in Jian carp [8] and grass carp [9]. However, no study has addressed the relationship between vitamin C and fish gill physical and immune barrier functions. It was reported that vitamin C deficiency could lead to deformation of gill filament cartilage in Channel Catfish (*Ictalurus punctatus*) [10]. Meanwhile, dietary vitamin C could significantly increase the vitamin C content in the gill of juvenile olive flounder (*Paralichthys olivaceus*) [11]. These data suggest that vitamin C may relate to the gill physical and immune barrier functions of fish, further studies are warranted to address these important questions.

Fish gill physical barriers are related to oxidative injury, apoptosis and intercellular tight junctional complexes (TJs) [Occludin, Claudins and zonula occludens (ZO)] [5]. Oxidative injury could be relieved by increasing non-enzymatic compounds contents and antioxidant enzymes activities in fish gill [12]. The activities of antioxidant enzymes are relying on their corresponding mRNA expressions, which could regulate by nuclear factor-E2-related factor 2 (Nrf2) in fish [13]. Apoptosis is related to caspase family in human [14]. Intercellular TJs were regulated by myosin light chain kinase (MLCK), and up-regulation of MLCK could disturb TJs in fish [15]. However, up to now, there is no information about the effects of vitamin C on the oxidative injury, apoptosis, TJs and their possible molecular mechanisms in fish gill. Studies have shown that, vitamin C could increase fish serum and liver antioxidant enzyme superoxide dismutase (SOD) activity [7,16]. It was reported that vitamin C could improve intestinal iron uptake in human [17]. Iron could activate Nrf2 signaling pathway in mice [18]. In addition, vitamin C could up-regulate heat shock protein 70 (HSP70) mRNA level in Wuchang bream (*Megalobrama amblycephala* Yih) [16]. In Atlantic salmon (*Salmo salar*), HSP70 could prevent caspase-3 activation [19]. Furthermore, vitamin C could decrease the content of tumor necrosis factor α (TNF-α) in mice [20]. In Caco-2 cells, TNF-α could up-regulate MLCK gene expression [21]. Therefore, these data suggest a possible correlation between vitamin C and oxidative injury, apoptosis, TJs and their possible signaling pathways in fish gill, which remains to be elucidated.

The immune barriers of fish gill largely depend on antibacterial compounds, such as lysozyme (LA), acid phosphatase (ACP), complement and antimicrobial peptides [3]. Furthermore, inflammatory response, mediated by cytokines, plays a key role in fish gill immune barriers [13]. The inflammation could be relieved by inhibiting nuclear factor-κB (NF-κB) and mammalian target of rapamycin (mTOR) signaling pathways in human [22,23]. However, studies have not addressed the effect of vitamin C on the antibacterial compounds, cytokines and its possible mechanism involved in NF-κB and TOR signaling pathways in fish gill. It was reported that vitamin C could inhibit p38 mitogen-activated protein kinase (p38 MAPK) in human [24]. In mice, inhibition of p38 MAPK could inhibit NF-κB signaling pathway activation [25]. In addition, vitamin C can protect tryptophan from oxidation [26]. Our lab previous study noted that tryptophan could down-regulate TOR mRNA level in fish intestine [27]. There may be a possible relationship between vitamin C and antibacterial compounds, cytokines and its possible mechanism in fish gill, which is valuable for investigation.

Dietary vitamin C requirement for the optimal growth of grass carp was determined to be 92.8 mg/kg diet in our previous study [9]. However, nutrient requirements of fish may vary with different sensitive indices [3]. Meanwhile, the metabolism of many nutrients is altered and the requirement for some is actually elevated due to increased catabolic and excretion processes in fish during the acute-phase immune response [28]. Therefore, it is valuable to determine the vitamin C requirements of grass carp based on the immune indices.

This study was a part of a larger study involved in the determination of the effects of vitamin C on the growth and head kidney, spleen and skin immunity of fish [9]. In fish, the growth performance is also closely related to the gill physical and immune barrier functions [3]. Thus, in this study, we hypothesize that optimal dietary vitamin C levels may enhance the gill physical and immune barrier functions to improve the global fish gill health status. *Flavobacterium columnare*, as an important pathogen, could adhere onto gill tissue, and cause of gill rot in fish [29]. To test this hypothesize, grass carp were challenged with *F. columnare*, and the related parameters of oxidative injury, apoptosis, TJs, antibacterial compounds and cytokines in the gill of grass carp were examined. Additionally, we further investigated the effects of vitamin C on the related signaling including Nrf2, apoptosis, MLCK, NF-κB and TOR in the gill of fish, which could provide partial theoretical evidence for the mechanisms of the vitamin C regulated gill physical and immune barrier functions for the maintenance of fish gill health. Meanwhile, the dietary vitamin C requirements according to gill physical and immune barrier functions indices were also evaluated, which may provide a reference for formulating the commercial feed of grass carp.

2. Materials and methods

2.1. Experimental diets preparation

Formulation of the basal diet was the same as in our previous study [9], and is shown in Table 1. Dietary protein and lipid were supplied by fish meal, casein and soybean protein concentrate, and fish oil and soybean oil, respectively. The dietary protein level was fixed at 300 g/kg diet, which was reported to be optimal for the