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Dietary riboflavin deficiency decreases immunity and antioxidant capacity, and changes tight junction proteins and related signaling molecules mRNA expression in the gills of young grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

This study investigated the effects of dietary riboflavin on the growth, gill immunity, tight junction proteins, antioxidant system and related signaling molecules mRNA expression of young grass carp (*Ctenopharyngodon idella*). Fish were fed six diets containing graded levels of riboflavin (0.63–10.04 mg/kg diet) for 8 weeks. The study indicated that riboflavin deficiency decreased lysozyme and acid phosphatase activities, and complement component 3 content in the gills of fish ($P < 0.05$). Moreover, riboflavin deficiency caused oxidative damage, which might be partly due to decrease copper, zinc superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase and glutathione-S-transferase activities and reduced glutathione content in the gills of fish ($P < 0.05$). Furthermore, the relative mRNA levels of antimicrobial peptides (liver expressed antimicrobial peptide 2 and Hepcidin), anti-inflammatory cytokines (interleukin 10 and transforming growth factor β1), tight junction proteins (Occludin, zonula occludens 1, Claudin-c and Claudin-3), signaling molecules (inhibitor of κBα, target of rapamycin and NF-E2-related factor 2) and antioxidant enzymes (copper, zinc superoxide dismutase and glutathione reductase) were significantly decreased ($P < 0.05$) in the gills of fish fed riboflavin-deficient diet. Conversely, the mRNA levels of pro-inflammatory cytokines (tumor necrosis factor α, interleukin 8, interferon γ2, and interleukin 1β), signaling molecules (nuclear factor kappa B p65, IκB kinase β, IκB kinase γ, Kelch-like-ECH-associated protein 1b and myosin light chain kinase) and tight junction protein Claudin-12 were significantly increased ($P < 0.05$) in the gills of fish fed riboflavin-deficient diet. In addition, this study indicated for the first time that young fish fed a riboflavin-deficient diet exhibited anorexia and poor growth. In conclusion, riboflavin deficiency decreased growth and gill immunity, impaired gill antioxidant system, as well as regulated mRNA expression of gill tight junction proteins and related signaling molecules of fish. Based on percent weight gain, gill lysozyme activity and reduced glutathione content, the dietary riboflavin requirements for young grass carp (275–722 g) were estimated to be 5.85, 7.39 and 6.34 mg/kg diet, respectively.

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1. Introduction

Riboflavin is an essential water-soluble vitamin for fish [1]. Our laboratory previous study reported that riboflavin deficiency decreased juvenile Jian carp (*Cyprinus carpio* var. Jian) growth performance [2]. Sutherland and Meyer [3] reported that fish growth was positively correlated with gill health. To our knowledge, fish gill health mainly depends on its immune [4] and barrier function [5]. However, no studies have addressed the relationship between riboflavin and fish gills. It was reported that riboflavin could enter the blood from the small intestine [6]. Monteiro et al. [7] reported that gill filaments composed of a highly complex vasculature and each lamella contained a single blood vessel entering and another leaving it. Therefore, dietary riboflavin may touch fish gill through the blood circulation and riboflavin deficiency decreased fish growth might associate with impairing gill immune and barrier function. This topic is valuable to further investigation.

In fish, the gill immune function partly depends on innate immunity [8]. However, information regarding the effects of riboflavin on innate immunity in fish gill is scarce. The antibacterial compounds, such as lysozyme (LA), acid phosphatase, complement and antimicrobial peptides play important roles in innate immunity of fish gill [9,10]. It was reported that fish gill owned mucosa associated lymphoid tissues, which harbor lymphocytes [4]. Trichet [11] reported that lymphocytes could produce antibacterial compounds in fish. Moreover, it has been reported that reduced glutathione play important role in lymphocyte replication in human [12]. In HepG2 cells, riboflavin could increase reduced glutathione content [13]. These data indicated that riboflavin might be able to increase gill reduced glutathione content, thereby improving gill innate immunity of fish, and this possibility was worthy of investigation. In fish, the innate immune response could trigger an inflammation, which was primarily mediated by inflammatory cytokines [14]. It has been reported that the production of inflammatory cytokines were regulated by nuclear factor κB (NF-κB) signaling pathway in bony fish [15] and target of rapamycin (TOR) signaling pathway in human cells [16]. However, no reports at present have addressed the effects of riboflavin on inflammatory cytokines production through NF-κB and TOR signaling pathways in fish. In mice, riboflavin could increase the expression of heat shock protein 25 [17]. Yi et al. [18] reported that overexpression of heat shock protein 25 could activate NF-κB signaling pathway in murine L929 cells. Meanwhile, riboflavin could inhibit p38 mitogen-activated protein kinase (p38 MAPK) activation in islet cells [19]. In murine embryonic fibroblasts, the activation p38 MAPK could induce TOR signaling pathway [20]. Above data indicated that riboflavin may affect the inflammatory cytokines production through NF-κB and TOR signaling pathways in fish, which need to be investigated.

In fish, gill health also relies on gill barrier function, which mainly made up of epithelial cells [21] and tight junctions (TJs) [22]. In fish gill, TJ complex consists of both transmembrane and cytosolic proteins, such as Occludin, Claudins and zonula occludens 1 (ZO-1) [23]. However, no study has focused on the effects of riboflavin on TJ proteins in animal. In mice, riboflavin could reduce the expression of inducible nitric oxide synthase [24]. It was reported that inducible nitric oxide synthase could disrupt the localization of ZO-1 and Occludin in the Ileal epithelium of mice [25]. These data indicated that riboflavin may have effects on TJ complex in fish gill, however, studies have not been conducted to investigate this. In addition, epithelial cells also play important role in gill barrier function of fish [21]. It was reported that gill was prone to oxidative damage, which could disturb structural integrity of epithelial cells [26]. Fish gills have developed antioxidant system, such as antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) to combat oxidative damage [27]. Lambertucci et al. [28] reported that antioxidant enzyme activities partly dependent on antioxidant enzyme genes transcription in rat muscle. Furthermore, antioxidant enzymes genes expression has proven to be regulated by NF-E2-related factor 2 (Nrf2), the key transcription factor in mice [29]. Recently, our laboratory was the first to clone the cDNA of Nrf2 (GenBank accession no. KF733814) of grass carp (*Ctenopharyngodon idella*), and demonstrated that dietary tryptophan could up-regulate copper, zinc superoxide dismutase (CuZnSOD) and GPx mRNA expression through increasing Nrf2 mRNA level, thereby increasing their activities in the intestine of grass carp [30]. However, no studies have investigated the effects of riboflavin on antioxidant enzymes gene expression through Nrf2, and subsequent regulated antioxidant enzymes activities in animal. In mice, riboflavin could reduce the production of plasma nitric oxide [24]. Liu et al. [31] reported that nitric oxide could increase Nrf2 gene expression in vascular smooth muscle cells of rat. Above data indicated that riboflavin might regulate antioxidant enzyme activities through modulating their gene expression, which may relate to Nrf2 signaling pathway in the gills of fish. This possibility is worth of investigation.

Grass carp is a broad distribution species over the world [32]. The dietary riboflavin requirement of grass carp has been evaluated in grass carp fingerling [33]. However, the nutrients requirements of fish may vary with different growth stage and different indices. It was reported that, in rainbow trout (*Oncorhynchus mykiss*), riboflavin requirement in fry (initial weight 2.00 g) was two times higher than that at beyond the fingerling stage (initial weight 60.00 g) [34]. Meanwhile, our lab previous study has found that, the *myo*-inositol requirement for juvenile Jian carp based on muscle protein carbonyl content was higher than that based on percent weight gain [35]. Therefore, it is valuable to investigate the dietary riboflavin requirements of young grass carp based on growth, immune and antioxidant indicators.

This study determined the effects of riboflavin on fish growth performance. Further, we for the first time investigated the effects of riboflavin on immune function, antioxidant system, and mRNA expression of tight junction proteins in the gills of young grass carp. To investigate the underling mechanisms, we firstly examined the effects of riboflavin on mRNA levels of signaling molecules, including TOR, NF-κB p65, inhibitor of κBα (IκBα), IκB kinase α (IKKα), IκB kinase β (IKKβ), IκB kinase γ (IKKγ), myosin light chain kinase (MLCK), Nrf2, Kelch-like-ECH-associated protein 1a (Keap1a) and Kelch-like-ECH-associated protein 1b (Keap1b) in the gills of young grass carp, which may provide partial theoretical evidence for the mechanisms of riboflavin-improved fish growth. In addition, the riboflavin requirements of young grass carp were also determined in this study, which may provide partial theoretical evidence for the commercial feed production of grass carp.

2. Materials and methods

2.1. Experimental diets preparation

Formulation of the basal diet is shown in Table 1. Fish meal (Pesquera Lota Protein Ltd., Lota, Chile), casein (Hulunbeier Sanyuan Milk Co., Ltd., Inner Mongolia, China) and gelatin (Rousselot Gelatin Co., Ltd., Guangdong, China) were used as dietary protein sources. Fish oil (CIA. Pesquera Camanchaca S.A., Santiago, Chile) and soybean oil (Kerry Oils & Grains Industrial Co., Ltd., Sichuan, China) were used as dietary lipid sources. The dietary protein level was fixed at 300 g/kg diet, which was reported to be optimum for the growth of grass carp, as described by Khan et al. [36]. Riboflavin (Sigma, St. Louis, MO, USA) was added to the basal

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