Fish & Shellfish Immunology 47 (2015) 289–301

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Folic acid deficiency impairs the gill health status associated with the NF-κB, MLCK and Nrf2 signaling pathways in the gills of young grass carp (*Ctenopharyngodon idella*)

Lei Shi [a, 1], Lin Feng [a, b, c, 1], Wei-Dan Jiang [a, b, c], Yang Liu [a, b, c], Jun Jiang [a, b, c], Pei Wu [a, b, c], Juan Zhao [a], Sheng-Yao Kuang [d], Ling Tang [d], Wu-Neng Tang [d], Yong-An Zhang [e], Xiao-Qiu Zhou [a, b, c, *]

[a] Animal Nutrition Institute, Sichuan Agricultural University, Chengdu 611130, China
[b] Fish Nutrition and Safety Production University Key Laboratory of Sichuan Province, Sichuan Agricultural University, Chengdu 611130, China
[c] Key Laboratory for Animal Disease-Resistance Nutrition of China Ministry of Education, Sichuan Agricultural University, Chengdu 611130, China
[d] Animal Nutrition Institute, Sichuan Academy of Animal Science, Chengdu 610066, China
[e] Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

ARTICLE INFO

Article history:
Received 17 April 2015
Received in revised form
11 September 2015
Accepted 11 September 2015
Available online 14 September 2015

Keywords:
Folic acid
Grass carp
Gills
Immune function
Barrier function

ABSTRACT

The aim of this study was to investigate the effect of dietary folic acid on fish growth, the immune and barrier functions of fish gills, and the potential mechanisms of these effects. Young grass carp (*Ctenopharyngodon idella*) were fed diets containing graded levels of folic acid at 0.10 (basal diet), 0.47, 1.03, 1.48, 1.88 and 3.12 mg kg^{-1} diet for 8 weeks. The results showed that acid phosphatase and lysozyme activities and the complement component 3 content in fish gills decreased with folic acid deficiency ($P < 0.05$). Folic acid deficiency up-regulated liver-expressed antimicrobial peptide 1, interleukin 1β, interleukin 8, tumor necrosis factor α, nuclear factor κB p65, IκB kinase α (IKK-α), IKK-β and IKK-γ gene expression. Folic acid deficiency down-regulated interleukin 10, transforming growth factor β, IκB and target of rapamycin gene expression in fish gills ($P < 0.05$). These results showed that limited folic acid decreased fish gill immune status. Furthermore, folic acid deficiency down-regulated claudin-b, claudin-c, claudin-3, occludin and zonula occludens 1 gene expression, whereas folic acid deficiency up-regulated claudin-12, claudin-15, myosin light chain kinase and p38 mitogen activated protein kinase gene expression in fish gills ($P < 0.05$). These results suggested that folic acid deficiency disrupted tight junction-mediated fish gill barrier function. Additionally, folic acid deficiency increased the content of reactive oxygen species, protein carbonyl and malondialdehyde (MDA); Mn superoxide dismutase activity and gene expression; and Kelch-like-ECH-associated protein 1a (Keap1a) and Keap1b gene expression ($P < 0.05$). Conversely, folic acid deficiency decreased Cu/Zn superoxide dismutase, catalase, glutathione peroxidase, glutathione s-transferases and glutathione reductase activities and gene expression as well as NF-E2-related factor 2 gene expression in fish gills ($P < 0.05$). All of these results indicated that folic acid deficiency impaired fish gill health status via regulating gene expression of cytokines, tight junction proteins, antioxidant enzymes, NF-κB p65, MLCK and Nrf2. Based on percent weight gain, LZ activity and MDA content in the gills, the dietary folic acid requirements for young grass carp were 1.60, 2.07 and 2.08 mg kg^{-1}, respectively.

1. Introduction

The gill is an important organ that plays a vital role in respiration, homeostatic equilibrium and immune response in fish [1]. As one of the major lymphoid tissues, the fish gill harbors mechanisms to avoid invaders from waterborne contaminants [2]. It was reported that fish gill disturbance results in an impairment of gill

* Corresponding author. Animal Nutrition, Sichuan Agricultural University, Chengdu 611130, Sichuan, China.
E-mail addresses: xqzhouqq@tom.com, zhouxq@sicau.edu.cn (X.-Q. Zhou).
[1] These two authors contributed to this work equally.

http://dx.doi.org/10.1016/j.fsi.2015.09.023
1050-4648/

immunity, leading to retarded growth [3] and even high mortality [4]. Therefore, maintaining gill health status is of the utmost importance in fish. To date, limited investigations have shown the effect of nutrients on gill health. Studies from our laboratory showed that arginine deficiency [5] or *myo*-inositol deficiency [6] had a negative effect on fish gill health. Folic acid is an important water-soluble vitamin for fish [7]. It has been confirmed that folic acid could be carried by transferrin in humans [8]; Meanwhile, fish gills contain the transferrin receptor [9]. These observations suggested that folic acid might be transferred into fish gills via the transferrin transfer. In addition, folic acid deficiency resulted in poor growth of grouper (*Epinephelus malabaricus*) [10]. Furthermore, it was reported that fish growth is positively correlated with gill health status [11]. These data indicated that a possible correlation exists between folic acid and gill health in fish, which requires investigation.

In general, fish gill health status is strongly correlated with immune function, which is dependent on the immune response [12]. In fish gills, the immune response is closely associated with antibacterial compounds, such as acid phosphatase (ACP), lysozyme, complement component 3 (C3) and antibacterial peptides [13,14], and cytokines, such as interleukin-8 (IL-8) and transforming growth factor β (TGF-β) [5]. Study in loach (*Paramisgurnus dabryanus*) showed that the production of cytokines could be involved in nuclear factor κB (NF-κB) signaling pathway [15]. However, no study has addressed the role of folic acid on cytokines; thus, it is unknown whether cytokine regulation by folic acid is associated with the mediation of the NF-κB pathway in fish. Study in human showed that folic acid decreased plasma C-reactive protein (CRP) level [16]. Meanwhile, CRP induced IL-1β expression in human monocytes [17]. Furthermore, it was reported that CRP activated NF-κB in human vascular endothelial cells [18]. These data indicated that folic acid might affect the regulation of cytokine expression by modulating NF-κB signaling in fish, which requires investigation.

In addition to the immune function, fish gill health is also closely associated with gill barrier function [19]. It is well known that gill barrier in fish is partially formed by tight junction proteins [20], which are mainly composed of zonula occludens 1 (ZO-1), occludin and claudins [21]. Furthermore, it has been reported that myosin light chain kinase (MLCK) is essential to the regulation of tight junction proteins in mice [22]. However, there are no studies on the effect of folic acid on tight junction proteins in fish, and whether MLCK is involved in this process is unknown. In murine macrophages, folic acid suppressed p38 mitogen activated protein kinase (p38 MAPK) induction by lipopolysaccharide [23]. Furthermore, inhibition of p38 MAPK prevented MLCK expression in mice intestine [24]. These observations indicated a possible correlation between folic acid and the tight junction proteins involved in MLCK signal molecule in fish; however, the conjecture requires confirmation.

Apart from the tight junction proteins, fish gill barrier functions are also strongly related to gill structural integrity [25]. Previous studies have shown that gill structural integrity is concerned in antioxidant capacity in fish [5]. Antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidases (GPx), play important roles in the antioxidant capacity of fish [26]. Furthermore, the antioxidant enzyme activity is partly dependent on enzyme gene transcription in rat [27]. Jiang et al. [28] indicated that NF-E2-related factor 2 (Nrf2) is a pivotal transcription factor that induces the transcription of antioxidant enzymes gene in fish. However, the effect of folic acid on the expression of antioxidant enzymes gene, and whether this event is associated with Nrf2 signaling in animals is not known. It was reported that folic acid increased nitric oxide (NO) production in mice microvascular endothelial cells [29]. *In vitro*, NO up-regulated Nrf2 gene expression in rat vascular smooth muscle cells [30]. These data indicated that folic acid might mediate antioxidant enzyme activities, which may be involved in the regulation of their gene expression through Nrf2 signaling in fish. This possibility requires further investigation.

Grass carp (*Ctenopharyngodon idella*) is widely cultivated in the world as an edible fish [31]. To date, the dietary folic acid requirement has been evaluated in juvenile grass carp [32]. However, the nutrient requirements may be different at different growth stages in fish [33]. Additionally, in fish, to maintain adequate immunity, the nutrient requirement is higher than that for normal growth [34]. Therefore, it is necessary to study folic acid requirements of young grass carp based on gill health indicators and growth.

From the foregoing, we preliminary investigated effect of folic acid on the innate immune response, tight junctions and antioxidant capacity, and made further efforts to test the potential mechanism for folic acid deficient diet affects fish gill immune and barrier functions, including the NF-κB, MLCK and Nrf2 signaling pathways. The results provide partial theoretical evidence for the effect of folic acid on fish gill health status. Moreover, the optimum dietary folic acid requirements of young grass carp based on gill health indicators and growth were estimated, which may be used in formulating commercial feeds for the intensive culture of grass carp.

2. Materials and methods

2.1. Experimental diets

The formulation of the experiment diet is given 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 main protein sources. Fish oil (CIA. Pesquera Camanchaca S.A., Santiago,

Table 1
Composition and nutrients content of basal diet.

Ingredients	g kg^{-1}	Nutrients content	g kg^{-1}
Fishmeal	37.50	Crude protein[d]	293.20
Casein	248.10	Crude lipid[d]	45.40
Gelatin	75.00	n-3[e]	10.00
DL-Met (99%)	1.40	n-6[e]	10.00
α-starch	240.00	Available phosphorus[e]	6.00
Corn starch	231.20		
Fish oil	25.00		
Soybean oil	18.90		
Cellulose	50.00		
$Ca(H_2PO_4)_2$	22.40		
Vitamin premix[a]	10.00		
Mineral premix[b]	20.00		
Folic acid premix[c]	15.00		
Choline chloride (60%)	5.00		
Ethoxyquin (30%)	0.50		

[a] Vitamin premix (g kg^{-1} premix): retinyl acetate (500 000 IU g^{-1}), 2.40; cholecalciferol (500 000 IU g^{-1}), 0.40; D,L-α-tocopherol acetate (50%), 12.54; menadione (23%) 0.79; thiamine nitrate (98%), 0.04; calcium-D-pantothenate (98%), 2.43; pyridoxine hydrochloride (98%), 0.59; cyanocobalamin (1%), 0.81; niacin (99%), 2.17; D-biotin (2%), 4.91; mesoinositol (99%), 19.19; riboflavin (80%), 0.55; ascorhyl acetate (93%), 7.16. All ingredients were diluted with corn starch to 1 kg.

[b] Mineral premix (g kg^{-1} premix): $MgSO_4 \cdot H_2O$, 56.200; $FeSO_4 \cdot H_2O$, 22.900; $CuSO_4 \cdot 5H_2O$, 0.020; $ZnSO_4 \cdot H_2O$, 0.630; $MnSO_4 \cdot H_2O$, 1.650; KI, 0.070; $NaSeO_3$, 0.004. All ingredients were diluted with corn starch to 1 kg.

[c] Folic acid premix (mg kg^{-1} premix): premix was added to obtain graded levels of folic acid. The final folic acid concentrations in each experimental diet were determined to be 0.10, 0.47, 1.03, 1.48, 1.88, 3.12 mg kg^{-1} diet, respectively.

[d] Crude protein, crude lipid and total phosphorus contents were measured value.

[e] Available phosphorus, n-3 and n-6 contents were calculated according to NRC (2011).