Fish & Shellfish Immunology 51 (2016) 405–419

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Immunity decreases, antioxidant system damages and tight junction changes in the intestine of grass carp (*Ctenopharyngodon idella*) during folic acid deficiency: Regulation of NF-κB, Nrf2 and MLCK mRNA levels

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], 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 27 October 2015
Received in revised form
19 February 2016
Accepted 21 February 2016
Available online 8 March 2016

Keywords:
Folic acid
Grass carp
Intestine
Immune barrier
Physical barrier

ABSTRACT

This investigation used the same growth trial as the previous study, which showed that folic acid deficiency retarded growth in young grass carp (the percent weight gain of Groups 1–6 were 102.32 ± 3.41%, 137.25 ± 10.48%, 179.78 ± 3.95%, 164.33 ± 3.21%, 143.35 ± 8.12% and 115.28 ± 2.66%) [1]. In the present study, we investigated the effects of dietary folic acid on the immune response, antioxidant status and tight junctions in the intestine of young grass carp (*Ctenopharyngodon idella*). A total of 540 young grass carp were fed diets containing graded levels of folic acid at 0.10, 0.47, 1.03, 1.48, 1.88 and 3.12 mg kg^{-1} diet for 8 weeks. The results indicated that acid phosphatase and lysozyme activities, and the complement component 3 content in the proximal intestine (PI), mid intestine (MI) and distal intestine (DI) were decreased with folic acid deficiency (0.1 mg kg^{-1}) ($P < 0.05$). Folic acid deficiency (0.1 mg kg^{-1}) up-regulated interleukin 1β, interleukin 8, tumor necrosis factor α, nuclear factor κB p65 (NF-κB p65), IκB kinase α (IKK-α), IKK-β and IKK-γ gene expression, meanwhile down-regulated interleukin 10, transforming growth factor β, IκB and target of rapamycin gene expression in the PI, MI and DI ($P < 0.05$). These data suggested that folic acid deficiency decreased fish intestinal innate immune function may be partly contributed to the regulation of NF-κB p65 pathway. Moreover, the activities and corresponding gene expression of glutathione content, Cu/Zn superoxide dismutase, catalase, glutathione peroxidase, glutathione s-transferases and glutathione reductase in fish intestine were depressed by deficient folic acid diet (0.1 mg kg^{-1}) ($P < 0.05$). Furthermore, folic acid deficiency (0.1 mg kg^{-1}) down-regulated NF-E2-related factor 2 (Nrf2) gene expression, up-regulated Kelch-like-ECH-associated protein 1a (Keap1a) and Keap1b gene expression in fish intestine ($P < 0.05$). These data indicated that deficient folic acid diet damaged fish intestinal antioxidant capacity partly by regulating Nrf2/Keap1 pathway. Additionally, folic acid deficiency (0.1 mg kg^{-1}) down-regulated claudin-b, claudin-c, claudin-3, occludin and zonula occludens 1 gene expression; whereas folic acid deficiency (0.1 mg kg^{-1}) up-regulated claudin-12, claudin-15, myosin light chain kinase (MLCK) and p38 mitogen activated protein kinase (p38 MAPK) gene expression in the PI, MI and DI ($P < 0.05$), suggesting that folic acid deficiency may damage fish intestinal tight junctions associated with the mediation of MLCK and p38 MAPK gene expression. In conclusion, folic acid deficiency (0.1 mg kg^{-1}) impaired fish intestinal immunity, antioxidant capacity and tight junctions.

1. Introduction

The intestine is an important organ that occupies central roles in the digestion/absorption of nutrients and immunity of fishes [2].

* 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.2016.02.029
1050-4648/

The intestine of fish contains many lymphoid cells, macrophages, eosinophilic and neutrophilic granulocytes [3]. The intestine also has extensive contact with the exterior environment, and therefore has the greatest chance of contact with foreign substances such as microorganisms, antigens, and toxic elements in food [4]. To protect the intestine against potentially dangerous microbes and some other toxic elements, fish have developed an intestinal mucosal immune system [5]. In general, this mainly consists of immune barrier and physical barrier [6]. Folic acid is an essential B vitamin for fish [7]. Data from our previous showed that folic acid deficiency decreased fish growth and feed efficiency (FE) [1]. In fish, FE is positively correlated with intestinal absorption ability, which may be related to the intestinal mucosal immune barrier and physical barrier [8]. Therefore, there may be a close relationship between folic acid and the fish intestinal immune barrier and physical barrier, which warrants further investigation.

Fish intestinal immune barrier is largely dependent on antibacterial compounds, such as acid phosphatase (ACP), lysozyme (LZ) and complement component 3 (C3) [9]. Main et al. [10] reported that folic acid participates in methionine synthesis in humans. In Jian carp (*Cyprinus carpio* var. Jian), methionine deficiency decreased LZ and ACP activities, as well as C3 content [11]. These findings indicated that folic acid deficiency may have negative effects on the fish intestinal antibacterial compounds. In fish, the decrease of LZ activity in intestinal mucosa induced inflammatory response [12], which is primarily mediated by cytokines, such as tumor necrosis factor α (TNF-α) and transforming growth factor-β (TGF-β) [13]. In addition, cytokines can be regulated by nuclear factor κB (NF-κB) signaling in mammals [14]. Unfortunately, no study exists on the role of folic acid in regulating cytokines and NF-κB in fish intestine. Folic acid significantly increased serum insulin-like growth factor-1 (IGF-1) level in rat embryos [15]. Moreover, IGF-1 induced Akt/mTOR pathway transcription in mice myoblasts [16]. Meanwhile, inhibition of mTOR promoted NF-κB expression in human endothelial cells [17]. These observations indicated a possible correlation between folic acid and NF-κB signaling in fish, which needs to be investigated.

Apart from the immune barrier, fish have also established a physical barrier in the intestinal mucosal immune system, which is firstly associated with enterocyte structure integrity [18]. Feng et al. [19] implied that intestinal structural integrity may be partly related to the antioxidant capacity in fish. A previous study showed that antioxidant enzymes play a vital role in the fish intestinal antioxidant system [20]. In addition, the antioxidant enzyme activities are partly related to gene transcription, which is regulated by the NF-E2-related nuclear factor 2 (Nrf2) signaling pathway in fish [21]. However, no reports at present have addressed the role of Nrf2 in regulating antioxidant enzyme gene expression by folic acid in fish intestine. Blom and Smulders [22] reported that folic acid deficiency increased homocysteine content in humans. Moreover, high levels of homocysteine down-regulated Nrf2 gene transcription in human lens epithelial cells [23]. These results indicated that there may be a relationship between folic acid and antioxidant enzyme gene expression associated with Nrf2 molecule in fish, which warrants further investigation. Oxidative stress also induced disruption of the tight junction protein-regulative barrier function in murine [24]. To our knowledge, tight junction proteins are mainly composed of claudin superfamily, occludin and ZO-1 [25], which regulated intestinal permeability in animal epithelia [26]. It has been confirmed that myosin light chain kinase (MLCK) regulated intestinal barrier function through the mediation of tight junction proteins in murine [27]. Moreover, it was reported that folic acid suppressed p38 mitogen activated protein kinases (MAPK) activity in mice macrophages [28]. Meanwhile, it was also found that inhibition of p38 MAPK prevented the increase of MLCK expression in mice intestine [29]. The above data indicated that folic acid may affect intestinal tight junction proteins related to MLCK molecule in fish, which warrants investigation.

This study used one growth trial from our previous study, which was a part of a larger study of folic acid improvements in fish gills immune and barrier function [1]. The objective of this study was to explore the effects of folic acid on the intestinal immune response, antioxidant status and tight junction proteins transcript abundance, as well as the gene expression of NF-κB p65, TOR, Nrf2, MLCK and p38 MAPK molecules. Although the optimum folic acid requirement of young grass carp has been estimated based on percent weight gain in our previous study [1], the requirements of nutrients may vary with sensitive indicators [30]. Additionally, malondialdehyde (MDA) could reflect intestinal health status [31], and MDA content was also used to define the nutrient requirement of fish [32]. Thus, the optimum dietary folic acid requirement of young grass carp based on intestinal MDA content was estimated, which may be used in formulating commercial feeds for the intensive culture of grass carp.

2. Materials and methods

2.1. Experimental diets

This study used the same growth trial as in our previous study, which showed that dietary folic acid deficiency retarded growth in young grass carp (The percent weight gain of Groups 1–6 were 102.32 ± 3.41%, 137.25 ± 10.48%, 179.78 ± 3.95%, 164.33 ± 3.21%, 143.35 ± 8.12% and 115.28 ± 2.66%. The food intake of Groups 1–6 were 414.69 ± 0.18 g, 520.38 ± 0.36 g, 636.54 ± 0.66 g, 596.60 ± 0.38 g, 521.53 ± 0.33 g and 423.03 ± 0.71 g) [1]. Formulation of the basal diet is presented in Table 1. The dietary protein level was fixed at 30%, which was reported to be optimum for the growth of grass carp, as described by Khan et al. [33]. Different

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).