Aquaculture 484 (2018) 13–27

Contents lists available at ScienceDirect

Aquaculture

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

Vitamin E deficiency depressed gill immune response and physical barrier referring to NF-kB, TOR, Nrf2 and MLCK signalling in grass carp (*Ctenopharyngodon idella*) under infection of *Flavobacterium columnare*

Jia-Hong Pan[a], Lin Feng[a,b,c], Wei-Dan 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,*], Yang Liu[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

Keywords:
Vitamin E
Grass carp (*Ctenopharyngodon idella*)
Immunity
Antioxidant capacity
Apoptosis
Tight junction
Gill

ABSTRACT

The aim of this study was to investigate the effect of graded levels of vitamin E (0–225 mg/kg) on gill immune response and physical barriers, and relative mRNA levels of signalling molecules in the gill of grass carp (*Ctenopharyngodon idella*) under infection of *Flavobacterium columnare*. The results indicated that compared with optimal vitamin E supplementation, vitamin E deficiency (1) increased the rate of gill rot morbidity and aggravated gill histopathological injuries following *F. columnare* infection ($P < 0.05$); (2) decreased LA and ACP activities, complement component 3, complement component 4 and IgM contents and down-regulated antimicrobial peptides (liver-expressed antimicrobial peptide-2A, -2B, hepcidin, β-defensin) and IgT mRNA levels ($P < 0.05$); (3) down-regulated the mRNA levels of anti-inflammatory cytokines and the signalling molecules inhibitory protein κBα, the target of rapamycin and ribosome protein S6 kinase 1 ($P < 0.05$) and up-regulated the mRNA levels of pro-inflammatory cytokines and the signalling molecules NF-κB p65, IκB kinase α (IKKα), IKKβ and eIF4E-binding protein 1 ($P < 0.05$); (4) decreased activities of anti-superoxide anion, Cu/ZnSOD, MnSOD, GPx, GST and GR, and down-regulated mRNA levels of those antioxidant enzymes and signalling molecule NF-E2-related factor 2 in the gills of fish ($P < 0.05$); (5) down-regulated the relative mRNA levels of B cell lymphoma-2 protein, and up-regulated the relative mRNA levels of caspase-2, -3, -7, -9, Bcl-2 associated X protein and apoptotic protease activating facter-1 ($P < 0.05$); (6) down-regulated the relative mRNA levels of tight junction proteins (occludin, zonula occludens-1, ZO-2, claudin-3, -b, -c and -11a) and up-regulated signalling molecule myosin light chain kinase mRNA levels ($P < 0.05$). In conclusion, vitamin E deficiency disrupted gill immune barriers and physical barrier function via impairing gill immunity and antioxidant capacity, inducing apoptosis and changing tight junction protein transcription abundances and the related signalling molecules in the gills of fish. The optimal vitamin E requirements for against gill rot morbidity of grass carp (266–1026 g) were estimated to be 139.56 mg/kg diet. Meanwhile, based on immune indicator (ACP activity) and antioxidant indicator (MDA content), the optimal vitamin E requirements for young grass carp were estimated to be 130.67 and128.53 mg/kg diet, respectively.

1. Introduction

Vitamin E is an indispensable nutrient required to maintain normal physiological functions in fish (Ortuño et al., 2000). Study in blue tilapia (*Oreochromis uureus*) showed that vitamin E deficiency led to muscle degeneration and fin hemorrhages (Roem et al., 1990). Our

Abbreviations: LA, lysozyme; ACP, acid phosphatase; LEAP, liver-expressed antimicrobial peptide; IL, interleukin; TGF, transforming growth factor; NF-κB, nuclear factor kappa B; IκBα, inhibitory protein κBα; TOR, target of rapamycin; S6K1, ribosome protein S6 kinase 1; 4EBP, eIF4E-binding protein; IKK, IκB kinase; ROS, reactive oxygen species; MDA, malonaldehyde; PC, protein carbonyl; SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase; GSH, reduced glutathione; GR, glutathione reductase; GST, glutathione S-transferase; Nrf2, NF-E2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; Bcl-2, B cell lymphoma-2 protein; Bax, Bcl-2 associated X protein; Apaf1, apoptotic protease activating facter-1; MLCK, myosin light chain kinase

* Corresponding authors at: Animal Nutrition Institute, Sichuan Agricultural University, Chengdu 611130, Sichuan, China.
E-mail addresses: xqzhouqq@tom.com, zhouxq@sicau.edu.cn (X.-Q. Zhou), kyckgk@hotmail.com (Y. Liu).

http://dx.doi.org/10.1016/j.aquaculture.2017.10.028
Received 10 July 2017; Received in revised form 1 October 2017; Accepted 20 October 2017
Available online 23 October 2017
0044-8486/

recent study indicated that vitamin E deficiency resulted in growth depression in young grass carp (*Ctenopharyngodon idella*) (Pan et al., 2017). Sutherland and Meyer (2007) reported that fish growth was positively correlated with gill health, which is mainly depends on gill immune and barrier function (Li et al., 2015). Increased gill vitamin E concentration was observed in hybrid tilapia (*Oreochromis niloticus X O. aureus*) fed a diet adequate in vitamin E (Shiau and Hsu, 2002). However, no information is available concerning the effects of vitamin E on the immune response and physical barriers in the gills of fish.

In fish, the gill immune barrier mainly depends on its immune function, which is closely related to gill-associated lymphoid tissue (GIALT) (Koppang et al., 2015). GIALT has been reported to produce immune molecules which play an important role in gill immunity (Lazado and Caipang, 2014). Furthermore, the expression of immune molecules can be regulated by nuclear factor κB (NF-κB) (Liu et al., 2003) and the target the of rapamycin (TOR) signalling pathway in mammals (Powell et al., 2012). However, there are no reports concerning the effects of vitamin E on gill immune function and related mechanisms in fish. One study found that dietary vitamin E can stimulate the helper activity of T lymphocytes in mice (Tanaka et al., 1979). T lymphocytes play an essential role in gill immunity by producing immune molecules to defend against the bacteria that are present on mucosal surfaces (Gomez et al., 2013). In addition, vitamin E supplementation has been shown to increase the concentration of adiponectin in mice plasma (Landrier et al., 2009). Ajuwon and Spurlock (2005) reported that adiponectin can inhibit LPS-induced NF-κB activation in porcine macrophages. Furthermore, vitamin E can stimulate the production of cyclic adenosine monophosphate (cAMP) in human peripheral mononuclear cells (Salinthone et al., 2013). A study on rat thyroid cells showed that cAMP can activate the mammalian target of rapamycin (mTOR) signalling pathways (Blancquaert et al., 2010). The above data imply that vitamin E may affect fish gill immune and the mechanism may be associated with NF-κB and TOR signalling pathways. However, this requires thorough and systematic investigation.

In fish, gill health is also associated with the physical barrier, which largely depends on the structural integrity of epithelial cells and intercellular tight junctions (TJs) (Xu et al., 2016). The structural integrity of epithelial cells is related to apoptosis and antioxidant ability, which is reflected by the antioxidant content, antioxidant enzyme activity, antioxidant enzyme gene expression and the NF-E2-related factor 2 (Nrf2) pathway (Jiang et al., 2015a, 2015b). However, no studies have investigated the effects of dietary vitamin E on apoptosis, TJs complex, antioxidants and the Nrf2 pathway in the gill of fish. In mouse microglia, one study showed that vitamin E attenuated cyclooxygenase-2 (COX-2) protein synthesis (Egger et al., 2003). COX-2 can down-regulate TJ proteins in testis cells of rats (J et al., 2012). In addition, a study on rat vascular smooth muscle cells showed that vitamin E could increase nitric oxide formation (Ganz and Seftel, 1999). Buckley et al. (2003) reported that nitric oxide stimulated Nrf2 nuclear translocation in bovine vascular endothelium cells. Furthermore, Clément et al. (2010) reported that vitamin E could inhibit protein kinase C (PKC) activity in rat vascular smooth muscle. One study demonstrated that PKC could induce cell apoptosis in MDCK cells (Bivona et al., 2006). Based on these studies, vitamin E can be inferred to affect gill barrier function, which might be associated with TJs, antioxidant defence and cell apoptosis in fish. This topic is in valuable to further research.

This study is a part of a larger study aimed at determining the effects of vitamin E on fish growth and the healthy status using the same growth trial as the previous study (Pan et al., 2017). Here, we aim to explore the effect of dietary vitamin E on gill immunological functions, physical barrier and related signalling factors, which could provide a systemic understanding of vitamin E with respect to gill health in fish. Additionally, the dietary optimal vitamin E levels based on different indicators for young grass carp were also estimated, which may provide a reference for formulating the commercial feed of grass carp.

Table 1
Diet formulation and composition.

Ingredients	g/kg	Nutrients content	%
Fish meal	155.00	Crude protein[d]	29.08
Casein	38.00	Crude lipid[d]	3.30
soybean protein concentrate	253.00	Ash[d]	6.03
DL-Met(99%)	4.00	n-3 + n-6[e]	1.50
α-starch	240.00	Available phosphorus[e]	0.84
Corn starch	163.76		
Linseed oil	16.93		
Cellulose	50.00		
$Ca(H_2PO_4)_2$	28.81		
Vitamin premix (vitamin E free)[a]	10.00		
Mineral premix[b]	20.00		
Vitamin E premix[c]	10.00		
Choline chloride (50%)	10.00		
Ethoxyquin (30%)	0.50		

[a] Per kilogram of vitamin premix (g/kg): retinyl acetate (500,000 IU/g), 2.10; cholecalciferol (500,000 IU/g), 0.40; menadione (22.9%), 0.83; cyanocobalamin (1%), 0.94; D-biotin (2%), 0.75; folic acid (95%), 0.42; thiamine nitrate (98%), 0.11; ascorhyl acetate (95%), 4.31; niacin (99%), 2.58; meso-inositol (98%), 19.39; calcium-D-pantothenate (98%), 2.56; riboflavin (80%), 0.63; pyridoxine hydrochloride (98%), 0.62. All ingredients were diluted with corn starch to 1 kg.

[b] Per kilogram of mineral premix (g/kg): $MnSO_4 \cdot H_2O$ (31.8% Mn), 1.8900; $MgSO_4 \cdot H_2O$ (15.0% Mg), 200.0000; $FeSO_4 \cdot H_2O$ (30.0% Fe), 24.5700; $ZnSO_4 \cdot H_2O$ (34.5% Zn), 8.2500; $CuSO_4 \cdot 5H_2O$ (25.0% Cu), 0.960; KI (76.9% I), 0.0668 g; Na_2SeO_3 (44.7% Se), 0.0168. All ingredients were diluted with corn starch to 1 kg.

[c] Premix was added to obtain graded levels of vitamin E and the amount of corn starch was reduced to compensate. The final vitamin E concentrations in each experimental diet were determined to be 4.93 (un-supplemented), 45.07, 90.62, 135.54, 180.29 and 225.36 mg/kg diet, respectively.

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

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

2. Materials and methods

2.1. Experimental diet preparation

The diets formulation and composition are shown in Table 1. Fish meal, casein and soybean protein concentrate were used as dietary protein sources. Linseed oil was utilized as a dietary lipid source. The dietary protein level was fixed at 300 g/kg diet, which was reported to be optimal for the growth of grass carp, as described by Khan et al. (2004). The vitamin premix did not contain vitamin E was provided similar to Lee and Shiau (2004). Different concentrations of dl-α-tocopherol acetate were added to a basal diet to constitute the six levels of 0 (un-supplemented), 45, 90, 135, 180 and 225 mg/kg diet at the expense of small amounts of corn starch. The basal diet without addition of dl-α-tocopherol acetate was referred to as the control diet. All ingredients were mixed and pelleted as previously described by Li et al. (2014). After drying, the diets were stored at − 20 °C until used. The dietary vitamin E concentrations of the six diets were analyzed by colorimetric method to be 4.93 (un-supplemented), 45.07, 90.62, 135.54, 180.29 and 225.36 mg/kg diet, respectively, as described by Desai (1984).

2.2. Feeding trial

The procedures used in this study were approved by the University of Sichuan Agricultural Animal Care Advisory Committee. Grass carp were obtained from fisheries (Sichuan, China). Prior to experimentation, the apparently healthy fish were carried out and several randomly-selected fish were microbiologically examined. The fish were found to be free of parasite infestations and *F. columnare* was not isolated from any fish. The fish were acclimated to the experimental environment for four weeks, according to Li et al. (2014). Then, 540 fish (mean weight 266.39 ± 0.33 g) were randomly assigned to 18 experimental cages (1.4 × 1.4 × 1.4 m^3) at an equal stocking rate of 30 fish per cage and