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Dietary choline deficiency and excess induced intestinal inflammation and alteration of intestinal tight junction protein transcription potentially by modulating NF-κB, STAT and p38 MAPK signaling molecules in juvenile Jian carp

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ABSTRACT

This study investigated the effects of choline on intestinal mucosal immune and the possible mechanisms in fish by feeding juvenile Jian carp (*Cyprinus carpio* var. Jian) with graded levels of dietary choline (165–1820 mg/kg diet) for 65 days. The results firstly showed that choline deficiency induced inflammatory infiltration in the proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of fish. Meanwhile, compared with the optimal choline group, choline deficiency decreased the activities of lysozyme and acid phosphatase, contents of complement 3 and IgM in the intestine, downregulated the mRNA levels of antimicrobial peptides (liver-expressed antimicrobial peptide (LEAP) 2A and defensin-3 in the PI and MI, LEAP-2B and hepcidin in the PI, MI and DI), anti-inflammatory cytokines (interleukin (IL) 10 and transforming growth factor β2 in the PI, MI and DI), and signaling molecule IκB in the PI, MI and DI; while upregulated the mRNA levels of pro-inflammatory cytokines (IL-6a and tumor necrosis factor α in the MI and DI, interferon γ2b in the PI and MI, IL-1β and IL-6b in the PI, MI and DI), and signaling molecules (Toll-like receptor 4 in the MI, myeloid differentiation primary response 88 in the PI and MI, Janus kinase 3 and tyrosine kinase 2 in the MI and DI, nuclear factor kappa B (NF-κB), signal transducers and activators of transcription (STAT) 4 and STAT5 in the PI, MI and DI) of juvenile Jian carp, further indicating that choline deficiency caused inflammation and immunity depression in the intestine of fish. But choline deficiency decreased the PI IL-6a mRNA level, and increased the DI LEAP-2A and defensin-3 mRNA levels with unknown reasons. Furthermore, dietary choline deficiency downregulated mRNA levels of tight junction (TJ) proteins (claudin 3c in the PI and MI, claudin 7, claudin 11 and occludin in the PI, MI and DI) and signaling molecule mitogen-activated protein kinases p38 in the PI, MI and DI of juvenile Jian carp, whereas upregulated the mRNA levels of claudin 3b in the MI and DI, and claudin 3c in the DI. Moreover, the excessive choline exhibited negative effects on intestinal immunity and TJ proteins that were similar to the choline deficiency. In summary, dietary choline deficiency or excess caused the depression of intestinal mucosal immune by inducing inflammation and dysfunction of the intestinal physical barrier, and regulating related signaling molecules of fish.

1. Introduction

The intestine is an important site for nutrient digestion and absorption in fish, especially in stomachless species, and an important barrier for preventing entrance of antigens and pathogens in fish [1]. It was reported that the impaired intestinal barrier

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function resulted in increasing of disease susceptibility in Atlantic salmon (*Salmo salar* L.) [2]. Therefore, the intestinal barrier function is of outmost importance to maintain health of fish. Recently, the dietary nutrition stimulus is considered as one of the major strategies to increase intestinal barrier function in fish. Study from our laboratory firstly found that several vitamins such as thiamin [3], riboflavin [4], niacin [5] and folic acid [6] improved the intestinal barrier in grass carp (*Ctenopharyngodon idella*). Choline is considered to be an essential vitamin for fish [7]. In our previous study, we found that choline improved the intestinal digestive function [8], and enhanced the diseases resistance and systemic immunity of juvenile Jian carp (*Cyprinus carpio* var. Jian) [9]. However, whether choline could improve the intestinal barrier function of fish is unclear.

Fish intestinal immune barrier is an important part of the intestine barrier line, and consists of antibacterial compounds including lysozymes, complement factors and antimicrobial peptides (AMPs) [10]. Meanwhile, study from our laboratory found that intestinal immune barrier was impaired by the intestinal inflammatory disorders which were accompanied with upregulated pro-inflammatory cytokines and decreased anti-inflammatory cytokines in fish [11]. Nuclear factor kappa B (NF-κB), and signal transducers and activators of transcription (STAT) are important transcriptional activators that regulate cytokines transcription [12]. NF-κB can be regulated by the inhibitory protein IκB [13], and the upstream signaling molecules Toll-like receptor 4 (TLR4) and myeloid differentiation primary response 88 (MyD88) in mammal [14]. STAT can be activated by the upstream signaling molecule Janus kinases (JAKs) [15]. To date, no information concerned about the effect of choline on the intestinal immune barrier and the possible underlying mechanisms in fish. Our previous study showed that choline enhanced serum lysozyme activity and complement 3 (C3) content, decreased pro-inflammatory cytokines interleukin (IL) 1β and tumor necrosis factor (TNF) α mRNA levels while increased anti-inflammatory cytokines IL-10 mRNA levels in spleen and head kidney of juvenile Jian carp [9]. Furthermore, choline is the precursor for biosynthesis of acetylcholine (ACh) which is the cholinergic neurotransmitter [16]. In rat, a cholinergic agonist induced the secretion of intestinal lysozyme and defensin [17]. Stimulation of the vagus nerve decreased the IL-6 levels and activated STAT3 in mouse intestinal macrophages [18]. Choline also acts as an agonist of α7 nicotinic acetylcholine receptor (α7nAChR) in mice [19]. Activating α7nAChR attenuated the TNF-α level in lamina propria mononuclear cells of colitis mice [20], inhibited the NF-κB activation and increased the IκB levels in human microvascular endothelial cells [21]. Accordingly, choline may affect the intestinal immune barrier potentially by NF-κB and STAT signaling pathways in fish, however, this warrants further study.

Fish intestinal physical barrier is another important part of intestinal barrier line, and composes of intestinal epithelial cells and the tight junction (TJ) [1]. Occludins and claudins are two main protein families found in TJs and form the backbone of the TJ [22]. Moreover, mitogen-activated protein kinases (MAPKs) have emerged as important regulators of the TJ in mammal [23]. In human gastric epithelial cells, inhibition of $p38^{MAPK}$ prevented the decrease of occludin expression and disruption of TJ [24]. Yet no literature data are available concerning the effect of choline on intestinal TJs and the related signaling pathway. As mentioned above, the important metabolite of choline, acetylcholine is the principle vagal neurotransmitter. In rabbit, vagal stimulation increased the intestinal epithelial permeability [25]. Moreover, our previous study showed that choline downregulated the TNF-α expressions in spleen of Jian carp [9]. In human colon carcinoma cells, TNF-α decreased the occludin expression [26]. In rat, choline inhibited the activation of $p38^{MAPK}$ in ventricular myocytes [27]. Thus, there might be a relationship between choline and the intestinal TJs and signaling molecule $p38^{MAPK}$, which warrants investigation.

This study was a part of a larger study that involved in exploring the effects of choline on fish growth and disease resistance, and used the same growth trial as we previously reported [8]. Our previous study showed that dietary choline improved the intestinal digestive function [8], the systemic immunity and disease resistance of fish [9]. Here, the present study was performed to investigate the effect of dietary choline on the mucosal immune components, cytokines, tight junction proteins and related signaling molecules in the intestine of juvenile Jian carp aiming to provide partial theoretical evidence for the mechanisms by which choline improved health of fish.

2. Material and methods

2.1. Diets and feeding trail

The present study used the same animal trial as our previous study [8]. Formulation of the basal diet is presented in Table 1. The basal diet was formulated to contain 315.3 g crude protein/kg diet and 44.0 g lipid/kg diet. Choline chloride (Sigma, St Louis, MO, USA) was added to the basal diet to form six experimental diets with graded levels of choline, i.e. 165 (choline deficiency), 310, 607, 896, 1167 and 1820 mg/kg diet, which was measured according to the method of Venugopal [28]. The diets were fan-dried at room temperature and stored at −20 °C until use as our previous study [8].

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Table 1
Composition and nutrients content of the basal diet.

Ingredients	g/kg diet	Nutrients content[a]	g/kg diet
Fish meal	20.0	Crude protein	315.3
Soybean protein concentrate	170.3	Crude lipid	44.0
Casein	180.3	Crude ash	48.1
Rice protein meal	33.5	Available phosphorus	6.6
Gelatin	38.2	n-3	10.0
DL-methionine (99%)	4.2	n-6	10.0
Thr (98.5%)	3.6	Methionine	11.3
Fish oil	26.8		
Soy bean oil	16.7		
α-starch	140.0		
Corn starch	276.8		
$Ca(H_2PO_4)_2$	19.1		
Choline-free vitamin premix[b]	10.0		
Trace mineral premix[c]	10.0		
Choline chloride premix[d]	30.0		
Ethoxyquin (30%)	0.5		
Cellulose	20.0		

[a] Crude protein, crude fat, crude ash, methionine and available phosphorus were measured value. n-3 and n-6 contents were calculated as our previous study described [8].

[b] Per kilogram of choline-free vitamin premix(g/kg): retinyl acetate (500,000 IU/g) 0.800 g, cholecalciferol (500,000 IU/g) 0.480 g, DL-α-tocopherol acetate (500 g/kg) 20.000 g, menadione (500 g/kg) 0.200 g, cyanocobalamin (100 g/kg) 0.010 g, D-biotin (200 g/kg) 0.500 g, folic acid (960 g/kg) 0.521 g, thiamin nitrate (980 g/kg) 0.104 g, ascorhyl acetate (920 g/kg) 7.247 g, niacin (980 g/kg) 2.857 g, inositol (980 g/kg) 52.857 g, calcium-D-pantothenate (980 g/kg) 2.511 g, riboflavine (800 g/kg) 0.625 g, pyridoxine hydrochloride (980 g/kg) 0.755 g. All ingredients were diluted with corn starch to 1 kg.

[c] Per kilogram of trace mineral premix (g/kg): $CuSO_4 \cdot 5H_2O$ (250.0 g/kg Cu) 1.201 g, KI (38.0 g/kg I) 2.895 g, $MnSO_4 \cdot H_2O$ (318.0 g/kg Mn) 4.089 g, $NaSeO_3$ (10.0 g/kg Se) 2.500 g, $FeSO_4 \cdot 7H_2O$ (197.0 g/kg Fe) 69.695 g, $ZnSO_4 \cdot 7H_2O$ (225.0 g/kg Zn) 21.640 g. All ingredients were diluted with $CaCO_3$ to 1 kg.

[d] Per kilogram of choline chloride premix (g/kg): each treatment group containing choline chloride 0 g, 5.5170 g, 17.2769 g, 29.0368 g, 40.7967 g and 64.3166 g, respectively. Each choline chloride mixture was diluted with corn starch to 1 kg.