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Low or excess levels of dietary cholesterol impaired immunity and aggravated inflammation response in young grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

The present study explored the effect of cholesterol on the immunity and inflammation response in the immune organs (head kidney, spleen and skin) of young grass carp (*Ctenopharyngodon idella*) fed graded levels of dietary cholesterol (0.041–1.526%) for 60 days and then infected with *Aeromonas hydrophila* for 14 days. The results showed that low levels of cholesterol (1) depressed the innate immune components [lysozyme (LZ), acid phosphatase (ACP), complements and antimicrobial peptides] and adaptive immune component [immunoglobulin M (IgM)], (2) up-regulated the mRNA levels of pro-inflammatory cytokines [interleukin 1β (IL-1β), IL-6, IL-8, IL-12p35, IL-12p40, IL-15, IL-17D, tumor necrosis factor α (TNF-α) and interferon γ2 (IFN-γ2)], partly due to the activated nuclear factor kappa B (NF-κB) signalling, and (3) down-regulated the mRNA levels of anti-inflammatory cytokines [IL-4/13B, IL-10, IL-11, transforming growth factor (TGF)-β1 and TGF-β2], partly due to the suppression of target of rapamycin (TOR) signalling in the immune organs of young grass carp. Interestingly, dietary cholesterol had no influences on the IκB kinase α (IKKα) and IL-4/13A mRNA levels in the head kidney, spleen and skin, the IL-1β and IL-12p40 mRNA levels in the spleen and skin, or the β-defensin-1 mRNA level in the skin of young grass carp. Additionally, low levels of cholesterol increased the skin haemorrhage and lesion morbidity. In summary, low levels of cholesterol impaired immunity by depressing the innate and adaptive immune components, and low levels of cholesterol aggravated the inflammation response via up-regulating the expression of pro-inflammatory cytokines as well as down-regulating the expression of anti-inflammatory cytokines partly through the modulation of NF-κB and TOR signalling in the immune organs of fish. Similar to the low level of cholesterol, the excess level of dietary cholesterol impaired immunity and aggravated inflammation response in the immune organs of fish. Finally, based on the percent weight gain (PWG), the ability against skin haemorrhage and lesions as well as the LZ activity in the head kidney and the ACP activity in the spleen, the optimal dietary cholesterol levels for young grass carp were estimated as 0.721, 0.826, 0.802 and 0.772% diet, respectively.

1. Introduction

With the increasing aquaculture industry, high levels of plant protein ingredients are currently used to replace fishmeal in aquafeeds [1], which have negative effect on non-specific immunity and growth in fishes [2,3]. These negative effect are partly related to the lack of some steroids, essential amino acids and vitamins in plant protein ingredients [4–7]. Cholesterol is a key sterol serving as a precursor to physiologically active compounds in animals [8]. Study have shown that low levels of dietary cholesterol decreased disease resistance in yellowtail (*Seriola quinqueradiata*) [9]. To our knowledge, disease resistance can be impacted by immunity, which relies on the immune response of immune organs (such as head kidney and spleen) in fish [10]. To date, there have only been fragmentary reports concerning the effect of cholesterol on the immune response of immune organs in fish. Only one study observed that low levels of cholesterol decreased the

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respiratory burst activity of macrophages and phagocytic activity of leucocytes in the head kidney of rainbow trout (*Oncorhynchus mykiss*) [11]. Therefore, in-depth studies should be performed to investigate the effect of cholesterol on the immune response of immune organs in fish.

The fish immune response is tightly correlated with innate immune components, such as lysozyme (LZ), acid phosphatase (ACP), complements (such as C3) and antimicrobial peptides (such as β-defensin) [12], as well as adaptive immune components, such as immunoglobulin M (IgM) [13]. However, there is no report investigating the effect of dietary cholesterol on innate and adaptive immune components in the immune organs of fish. Previous studies have confirmed that low-cholesterol diets decreased the plasma vitamin E level in rabbit [14]. In a previous study, we observed that vitamin E deficiency decreased the LZ and ACP activities as well as complement 3 (C3) contents in the head kidney and spleen of grass carp [15]. In addition, inadequate dietary cholesterol could decrease whole body lipid levels in juvenile turbot (*Scophthalmus maximus* L.) [16]. This previous study demonstrated that the low level of lipids reduced the IgM contents in the head kidney and spleen of grass carp [17]. Moreover, insufficient dietary cholesterol could decrease plasma calcium levels in rainbow trout [18]. In humans, calcium deficiency could down-regulate β-defensin-2 gene expression in oral epithelial cells [19]. The above observations indicate that cholesterol might influence the immune response of immune organs associated with innate and adaptive immune components in fish, which is worthy of further investigation.

Additionally, a cascade of cytokines is released as part of the innate immune components, which play a role in the immune response of fish [20]. Cytokines include pro-inflammatory cytokines (such as IL-6 and IL-8), which could be regulated by nuclear factor kappa B (NF-κB) in humans [21], and anti-inflammatory cytokines (such as IL-10), which could be modulated by mammalian target of rapamycin (mTOR) in mammals [22]. However, there are no studies investigating the effect and potential mechanisms of cholesterol on pro-/anti-inflammatory cytokines in fish. Cholesterol serves as a precursor of cortisol in animals [8]. Castro et al. (2011) demonstrated that cortisol caused the down-regulation of IL-6 and IL-8 gene expression and the up-regulation of IL-10 expression in rainbow trout [23]. In addition, high dietary cholesterol could cause vitamin C deficiency in guinea pigs [24]. In a previous study, we demonstrated that vitamin C deficiency activated NF-κB signalling in the head kidney of grass carp [25]. Moreover, in humans, evidence suggests that cholesterol depletion inhibited Akt phosphorylation in prostate epithelial cells [26], and the inhibition of Akt phosphorylation could suppress mTOR signalling in HEK293 cells [27]. Hence, these data suggest a potential relationship between dietary cholesterol and pro-/anti-inflammatory cytokines as well as its potential regulation mechanisms in fish, which awaits investigation.

The present study, is the first to investigate the effect of dietary cholesterol on the innate and adaptive immune components in the immune organs of fish. Additionally, we further investigated the influences of dietary cholesterol on the relevant signalling pathways, namely, NF-κB and TOR, which might reveal partial theoretical evidence for the mechanisms of the immune response in fish. Moreover, grass carp is a typical herbivorous finfish [28] and is one of the most important commercial freshwater fish species worldwide [29]. Thus, the optimal dietary cholesterol levels for young grass carp were also evaluated, which may provide a reference for formulating the commercial feeds of grass carp.

2. Materials and methods

2.1. Experimental diets and procedures

The ingredients and proximate composition of experimental diets are presented in Table 1. Six isonitrogenous and isoenergetic diets were formulated to contain increasing levels of cholesterol. The basal diet (C0) was formulated by using a combination of casein, gelatin and soybean protein concentrate as the primary protein sources; in addition, fish oil and soybean oil were used as the dominating lipid sources. The diet formulation was based on that reported in previous studies and was considered sufficient in terms of protein, lipids, n-3 and n-6, as well as available phosphorus to meet the requirements of young grass carp according to Xu et al. (2016) [30], Ni et al. (2016) [17], Zeng et al. (2015) [31] and Wen et al. (2015) [32]. The other five diets (C0.3, C0.6, C0.9, C1.2 and C1.5) were supplemented with 0.3, 0.6, 0.9, 1.2 and 1.5% cholesterol (purity ≥ 95%), at the expense of corn starch in the basal diet. The actual cholesterol contents of C0, C0.3, C0.6, C0.9, C1.2 and C1.5 diets were determined as 0.041, 0.334, 0.636, 0.932, 1.243 and 1.526%, respectively, according to Deng et al. (2013) [18], and the diets were prepared and stored at −20 °C.

Table 1
Ingredients and proximate composition of the experimental diets for young grass carp (*Ctenopharyngodon idella*).

	Dietary treatments[a]					
	C0	C0.3	C0.6	C0.9	C1.2	C1.5
Ingredients (g kg^{-1})						
Casein	160.00	160.00	160.00	160.00	160.00	160.00
Gelatin	56.00	56.00	56.00	56.00	56.00	56.00
Soybean protein concentrate	150.00	150.00	150.00	150.00	150.00	150.00
α-starch	230.00	230.00	230.00	230.00	230.00	230.00
Corn starch	246.00	243.00	240.00	237.00	234.00	231.00
Fish oil	29.40	29.40	29.40	29.40	29.40	29.40
Soybean oil	18.10	18.10	18.10	18.10	18.10	18.10
Cellulose	50.00	50.00	50.00	50.00	50.00	50.00
Vitamin premix[b]	10.00	10.00	10.00	10.00	10.00	10.00
Mineral premix[c]	20.00	20.00	20.00	20.00	20.00	20.00
Cholesterol (95%)	0.00	3.00	6.00	9.00	12.00	15.00
$Ca(H_2PO_4)_2$	15.30	15.30	15.30	15.30	15.30	15.30
Choline chloride (50%)	10.00	10.00	10.00	10.00	10.00	10.00
DL-Met (99%)	4.10	4.10	4.10	4.10	4.10	4.10
L-Trp (99.2%)	0.60	0.60	0.60	0.60	0.60	0.60
Ethoxyquin (30%)	0.50	0.50	0.50	0.50	0.50	0.50
Proximate composition						
DM (%)[d]	88.31	88.43	88.61	88.25	88.98	88.22
Crude protein (%)[d]	28.71	29.01	29.11	28.78	28.80	28.97
Crude lipid (%)[d]	4.50	4.62	4.46	4.54	4.58	4.67
(Omega-3 fatty acids) n-3 (%)[e]	1.04	1.04	1.04	1.04	1.04	1.04
(Omega-6 fatty acids) n-6 (%)[e]	0.96	0.96	0.96	0.96	0.96	0.96
Available phosphorus[e]	0.40	0.40	0.40	0.40	0.40	0.40
Total energy (MJ kg^{-1} DM)[d]	19.77	19.93	20.05	19.99	20.20	20.30
Cholesterol (%)[d]	0.041	0.334	0.636	0.932	1.243	1.526

[a] Dietary treatments: C0, basal diet; C0.3, 0.3% cholesterol; C0.6, 0.6% cholesterol; C0.9, 0.9% cholesterol; C1.2, 1.2% cholesterol; C1.5, 1.5% cholesterol.

[b] Per kilogram of vitamin premix (g kg^{-1}): retinyl acetate (500,000 IU g^{-1}), 0.39; cholecalciferol (500,000 IU g^{-1}), 0.40; DL-α-tocopherol acetate (50%), 23.23; menadione (22.9%), 0.83; cyanocobalamin (1%), 0.94; D-biotin (2%), 0.75; folic acid (95%), 0.42; thiamine nitrate (98%), 0.09; ascorhyl acetate (95%), 9.77; niacin (99%), 4.04; meso-inositol (98%), 19.39; calcium-D-pantothenate (98%), 3.85; riboflavin (80%), 0.73; pyridoxine hydrochloride (98%), 0.62. All ingredients were diluted with maize starch to 1 kg.

[c] Per kilogram of mineral premix (g kg^{-1}): $MnSO_4.H_2O$ (31.8% Mn), 2.6590; $MgSO_4.H_2O$ (15.0% Mg), 200.0000; $FeSO_4.H_2O$ (30.0% Fe), 12.2500; $ZnSO_4.H_2O$ (34.5% Zn), 8.2460; $CuSO_4.5H_2O$ (25.0% Cu), 0.9560; KI (76.9% I), 0.0650; Na_2SeO_3 (44.7% Se), 0.0168. All ingredients were diluted with maize starch to 1 kg.

[d] Dry matter (DM), crude protein, crude lipid and cholesterol contents, as well as total energy were measured values.

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