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Effect of choline on antioxidant defenses and gene expressions of Nrf2 signaling molecule in the spleen and head kidney of juvenile Jian carp (*Cyprinus carpio* var. Jian)

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ABSTRACT

The present work evaluates the effects of various levels of dietary choline on antioxidant defenses and gene expressions of Nrf2 signaling molecule in spleen and head kidney of juvenile Jian carp (*Cyprinus carpio* var. Jian). Fish were fed with six different experimental diets containing graded levels of choline at 165 (choline-deficient control), 310, 607, 896, 1167 and 1820 mg kg^{-1} diet for 65 days. At the end of the feeding trail, fish were challenged with *Aeromonas hydrophila* and mortalities were recorded over 17 days. Dietary choline significantly decreased malondialdehyde and protein carbonyl contents in spleen and head kidney. However, anti-superoxide anion and anti-hydroxyl radical activities in spleen and head kidney also decreased. Interestingly, activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR) in spleen, GPx activity in head kidney, and glutathione contents in spleen and head kidney were decreased with increase of dietary choline levels up to a certain point, whereas, activities of SOD, GST and GR in head kidney showed no significantly differences among groups. Similarly, expression levels of CuZnSOD, MnSOD, CAT, GPx1a, GPx1b and GR gene in spleen and head kidney were significantly lower in group with choline level of 607 mg kg^{-1} diet than those in the choline-deficient group. The relative gene expressions of Nrf2 in head kidney and Keap1a in spleen and head kidney were decreased with increasing of dietary choline up to a certain point. However, the relative gene expression of Nrf2 in spleen were not significantly affected by dietary choline. In conclusion, dietary choline decreased the oxidant damage and regulated the antioxidant system in immune organs of juvenile Jian carp.

1. Introduction

Choline was discovered to be an essential nutrient for fish [1]. In our previous study, we found that choline improved growth, digestive and absorptive capacities [2], and enhanced the diseases resistance, immune function, growth and development of spleen and head kidney of juvenile Jian carp (*Cyprinus carpio* var. Jian) [3]. The growth and development of immune organs are dependent on the structural integrity of cells [4], which is threatened by oxidative stress [5]. Oxidative stress occurs when reactive oxygen species (ROS) generation rate exceeds their removal rate, and it can cause peroxidation of unsaturated lipids in cell membranes, oxidation of proteins, DNA and steroid components, leading to further damage of the cell integrity and normal functions [6]. ROS are products of normal cellular metabolism in aerobic organisms [7]. Meanwhile, the spleen and head kidney contain several immune cells including lymphocytes, macrophages and granulocytes [8]. John et al. [9] reported that leucocytes from the head kidney of immunized Indian major carp (*Catla catla*) produced higher levels of superoxide anion. Stimulation of B lymphocytes induces a burst of ROS production in mice [10]. Furthermore, fish cellular membranes contain uniquely high levels of n-3 highly unsaturated fatty acids which are highly susceptible to attack by oxygen and other organic radicals [11]. Accordingly, the spleen and head kidney of fish are prone to

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suffer from oxidative damage. However, whether choline improved the growth of spleen and head kidney via regulating the antioxidant defense of immune organs in fish is unclear.

Fish evolved a number of sophisticated antioxidant defense mechanisms, including enzymatic and non-enzymatic antioxidant system, to reduce oxidative stress [6]. Enzymatic antioxidant system includes endogenous antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR), which are usually distributed within all tissues of vertebrates [12]. SODs are the first and most important line of antioxidant enzyme defense systems against ROS [13], and CAT plays an important role in attenuating the potential toxicity of hydroxyl radical [12]. GPx, GST and GR are important glutathione-dependent enzymes and able to counteract the peroxidative damage [14]. Moreover, a variety of non-enzymatic antioxidants are also capable of alleviating oxidative stress in fish [15]. Reduced glutathione (GSH) is the most abundant low-molecular-weight thiol and plays an important role in antioxidant defense in animal cells [16]. However, little information is available about the effect of choline on fish antioxidant ability. *In vitro* biochemical assays showed that choline inhibited increase of peroxide value in methyl linoleate hydroperoxide [17] and sardine oil [18]. Saito and Ishihara [18] reported that the hydroxyl amines groups in choline showed as decomposers of hydroperoxides *in vitro*. In rat, dietary choline decreased product of lipid peroxidation in kidney [19] and reduced product of protein oxidation in brain mitochondrial [20]. These data showed a possible relation between choline and antioxidant ability in fish. Furthermore, the antioxidant enzyme activities of tissues were correlated with the mRNA levels in rat [21]. Albright et al. [22] showed that choline deficiency decreased DNA synthetic activity in rat hepatocytes. Nevertheless, there is no information regarding the relationship between choline and the gene level of antioxidant enzymes in fish. DNA methylation plays an important role in the regulation of gene expression [23]. Choline is an important methyl donor [24], and choline deficiency reduced DNA methylation in human neuroblastoma cells [23]. Thus, choline may affect the immune function of fish through regulating the mRNA levels and activities of antioxidant enzymes in immune organs, which awaits characterization.

The induction of antioxidant enzyme genes are regulated by several cell signaling pathways and transcription factors [7]. Nuclear factor erythoid 2-related factor 2 (Nrf2) is a master regulator of the cellular antioxidant response. When cells are exposed to oxidative stress, Nrf2 dissociates from Kelch-like ECH-associated protein 1 (Keap1), translocates to the nucleus, and ultimately activates expression of antioxidant response elements-dependent genes in terrestrial animal [25]. Recent study from our laboratory found that Nrf2 and Keap1 also exist in carp and widely distribute in several organs including spleen and head kidney, and can be regulated by inositol [26]. To date, no information concerned about the effect of choline on Nrf2 signaling pathway. In mice, the choline-Met deficient diet caused lower hepatic glutathione and more lipid peroxidation in the Nrf2-null mice [27]. Furthermore, the choline-Met deficient diet strongly induced the Nrf2 expression in mice [28,29]. Thus, there may be a close relationship between choline and Nrf2, and Nrf2 may play an important role in modulating gene expression of antioxidant enzymes regulated by choline in fish, however, these warrant investigation.

This study, using the same growth trial as we previously reported [2], was part of a larger study that involved in investigation of the effects of choline on fish digestive and immune functions. Our previous study showed that dietary choline improved the immune function of fish [3]. Here, we aim to explore a possible mechanism that explains how choline improved immune function of fish by determining the effect of dietary choline on antioxidant response and gene expression of important oxidative stress sensor (Nrf2 and Keap1) in immune organs of juvenile Jian carp.

2. Materials and methods

2.1. Experimental diets and feeding management

The experimental diets and feeding management were the same as previously reported by Wu et al. [2]. As presented in Table 1, casein and soy protein concentrate, and fish oil and soybean oil were the main dietary protein and lipid sources respectively. Choline chloride (Sigma, St Louis, MO, USA) was supplied to the basal diet to providing graded levels of choline at final concentration of 165 (unsupplemented control, deficient group), 310, 607, 896, 1167 and 1820 mg kg^{-1} diet, which were determined according to the method of Venugopal [30]. After pelleted, the diets were fan-dried at room temperature and stored at −20 °C until used [31].

Feeding management followed the Guidelines for the Care and Use of Laboratory Animals of Animal Nutritional Institute, Sichuan Agricultural University. Juvenile Jian carp were obtained from Tong Wei Hatchery (Sichuan, China) and acclimated for one month before starting experiment. 1200 healthy fish with average initial weight 7.94 ± 0.03 g were randomly divided into 24 aquaria (90 $L \times 30\ W \times 40\ H$ cm) each of which was aerated continuously. Fish were fed to satiation with each of the six experimental diets six times daily from 1 to 30 days and four times daily from 31 to 65 days. Water was drained through biofilters and changed constantly. Water temperature and pH were 25 ± 1 °C and 7.0 ± 0.3, respectively. Dissolved oxygen was higher than 5 mg L^{-1}. A natural light and dark cycle was maintained.

Table 1
Composition and nutrients content of the basal diet.

Ingredients	g kg^{-1} diet	Nutrients content (g kg^{-1} diet)[a]	
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 according to NRC (1993) and Bell (1984).

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

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

[d] Per kilogram of choline chloride premix (g kg^{-1}): 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.