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Effect of dietary glutamine on growth performance, non-specific immunity, expression of cytokine genes, phosphorylation of target of rapamycin (TOR), and anti-oxidative system in spleen and head kidney of Jian carp (*Cyprinus carpio* var. Jian)

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Abstract This study was designed to investigate the effects of dietary glutamine on the growth performance, cytokines, target of rapamycin (TOR), and antioxidant-related parameters in the spleen and head kidney of juvenile Jian carp (*Cyprinus carpio* var. Jian). Fish were fed the basal (control) and glutamine-supplemented (12.0 g glutamine kg^{-1} diet) diets for 6 weeks. Results indicated that the dietary glutamine supplementation improved the growth performance, spleen protein content, serum complement 3 content, and lysozyme activity in fish. In the spleen, glutamine down-regulated the expression of the interleukin 1 and interleukin 10 genes, and increased the level of phosphorylation of TOR protein. In the head kidney, glutamine down-regulated the tumor necrosis factor α and interleukin 10 gene expressions, phosphorylated and total TOR protein levels, while up-regulated the transforming growth factor β2 gene expression. Furthermore, the protein carbonyl content was decreased in the spleen of fish fed glutamine-supplemented diet; conversely, the anti-hydroxyl radical capacity and glutathione content in the spleen were increased by glutamine. However, diet supplemented with glutamine did not affect the lipid peroxidation, anti-superoxide anion capacity, and antioxidant enzyme activities in the spleen. Moreover, all of these antioxidant parameters in the head kidney were not affected by glutamine. Results from the present experiment showed the importance of dietary supplementation of glutamine in benefaction of the growth performance and several components of the innate immune system, and the deferential role in cytokine gene expression, TOR kinase activity, and antioxidant status between the spleen and head kidney of juvenile Jian carp.

Keywords Glutamine · *Cyprinus carpio* var. Jian · Cytokine · Target of rapamycin · Antioxidant status · Immune organ

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Introduction

Glutamine, a conditionally essential amino acid, appears to be an important nutrient for fish (Cheng

et al. 2012). A previous study from our laboratory found that dietary glutamine supplementation improved growth of juvenile Jian carp (*Cyprinus carpio* var. Jian) (Lin and Zhou 2006). Growth of fish is often related to the immune capacity (Pohlenz et al. 2012). Recently, a few studies reported glutamine improved lysozyme activity in the kidney macrophages of red drum (*Sciaenops ocellatus*) (Cheng et al. 2011), hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) (Cheng et al. 2012), and serum of hybrid sturgeon (*Acipenser schrenckii* ♀ × *Huso dauricus* ♂) (Zhu et al. 2011), as well as complement 3 (C3) and 4 (C4) levels in the serum of hybrid sturgeon (Zhu et al. 2011). These results suggested the importance of dietary supplementation of glutamine in improving growth performance and eliciting positive changes to several components of the non-specific defense response in fish.

It is known that pro-inflammatory cytokines [e.g., tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1)] are released as part of the innate immune response in fish and mammals (Whyte 2007). However, when these pro-inflammatory cytokines are uncontrolled and excessive, they can lead to the tissue injury and dysfunction in rodent (Enomoto et al. 2002; Rothwell 2003). Fortunately, anti-inflammatory cytokines such as transforming growth factor β (TGF-β) and interleukin 10 (IL-10) can control the production of pro-inflammatory cytokines to limit extensive inflammatory responses in fish (Reyes-Cerpa et al. 2013). In terrestrial animals, glutamine has been demonstrated to modulate production of cytokines, but the differential regulation has been observed in various animals. For example, glutamine increased TNF-α production in the murine macrophages (Wells et al. 1999), while it did not change the TNF-α production in the mesenteric lymph node cells of weaned piglet (Johnson et al. 2006). However, in fish, whether glutamine can affect the cytokines has not yet been studied. To date, these cytokines have been cloned in carp (Fujiki et al. 2000; Saeij et al. 2003; Savan et al. 2003; Sumathy et al. 1997). However, it was showed that amino acid sequences are large differences between mammalian and fish. For example, carp IL-1β shows only 21.8–24.7 % amino acid identities to mammalian mature IL-1β, and lacks a signal sequence (Fujiki et al. 2000). Therefore, in fish, whether glutamine can affect the cytokines need further investigation.

The production of cytokines is regulated by intracellular signaling pathways in immune systems (Chi 2012). Recently, emerging evidence indicated that mammalian target of rapamycin (mTOR) signaling not only regulated the protein synthesis but also mediated the transcriptional controls of cytokine in immune cells of murine and humans (Weichhart and Saemann 2009). In human immune cells, it was found that mTOR suppressed the production of pro-inflammatory cytokines and promoted the production of anti-inflammatory cytokines (Weichhart et al. 2008). Nicklin et al. (2009) indicated that mTOR signaling is sensitive to the availability of amino acids in terrestrial animal. Previous studies from our laboratory had been the first to identify the TOR in Jian carp (Jiang 2009) and showed that dietary arginine regulated the relative gene expressions of TOR in the muscle, hepatopancreas, and intestine of Jian carp (Chen et al. 2012). However, whether glutamine-affected cytokines is related to TOR signaling in fish is still unclear. Glutamine is an important precursor for the synthesis of arginine in humans (Ligthart-Melis et al. 2008) and murine macrophages (Murphy and Newsholme 1998). Furthermore, glutamine can stimulate the transcription of argininosuccinate synthetase gene, the key enzyme of arginine synthesis in human colon adenocarcinoma cells (Brasse-Lagnel et al. 2003). Thus, in view of apparent relation between glutamine and arginine, we speculated that glutamine can affect the cytokines through the TOR signaling in fish, a theory that requires investigation.

The antioxidant defense is also effective in protecting against injury in the immune organs of fish (Dautremepuits et al. 2004; Dorval et al. 2003). The spleen and head kidney are important immune organ in fish, which contain highly polyunsaturated fatty acids prone to oxidation damage (Waagbø et al. 1995; Ellis 1998). In addition, the immune cell of kidney could produce large amounts of oxidative radicals to protect fish against pathogens (Cheng et al. 2012). However, excessive ROS can induce oxidative damage in the spleen (Yonar 2012) and head kidney (Fatima et al. 2000) of fish. Like all aerobic organisms, fish antioxidant defense against ROS-mediated cellular injury comprises several antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferases (GST), and glutathione reductase (GR), and non-enzymic antioxidants, such as glutathione (GSH)

(Martínez-Álvarez et al. 2005). It would appear that glutamine is particularly likely to play an important role in enzymic and non-enzymic antioxidant defense in the hepatopancreas and muscle of hybrid sturgeon (Zhu et al. 2011). Previous studies from our laboratory also showed that glutamine maintained the integrity of cells through decreasing and repair oxidative damage by increasing the GSH content and enzymic antioxidant capacity in the enterocytes and erythrocytes of carp (Chen et al. 2009; Hu et al. 2014; Li et al. 2013). However, Kubrak et al. (2012) confirmed that protection against oxidative injury differs from tissue to tissue in fish. Our previous study found that histidine improved the activity of GR in the hepatopancreas, whereas no difference in the intestine of Jian carp (Feng et al. 2013). In addition, Li et al. (2014) showed that thiamin increased SOD activity in the hepatopancreas and intestine, but it did not change the SOD activity in the muscle of Jian carp. However, whether glutamine can affect the antioxidant defense in the spleen and head kidney of fish has not yet been studied, which needs to be investigated.

Therefore, the objective of the present study was to elucidate the effects of glutamine on the growth performance, the gene expression of cytokines, TOR kinase activity as well as antioxidant status in the spleen and head kidney of fish, as a mean of providing a partial theoretical evidences for the effects of glutamine on the growth of fish.

Materials and methods

Experimental diets

Formulation of the basal diet is presented in Table 1. Fish meal (Pesquera Lota Protein Ltd., Villagrán Lota, Chile), gelatin (Rousselot Gelatin Co., Ltd., Guangdong, China), and casein (Hulunbeier Sanyuan Milk Co., Ltd., Inner Mongolia, China) were used as dietary protein sources. Fish oil (CIA. Pesquera Camanchaca S.A., Santiago, Chile) and soybean oil (Kerry Oils & Grains Industrial Co., Ltd., Sichuan, China) were used as dietary lipid sources. The basal diet contained 338.4 g crude protein kg^{-1} diet and 45.6 g lipid kg^{-1} diet. The glutamine-supplemented diet was derived from the basal diet by supplementing 12.0 g glutamine (Sigma, St. Louis, MO, USA) kg^{-1} diet, which was the optimal dose for the growth of juvenile Jian carp according to our previous laboratory study (Lin and Zhou 2006). The basal diet was made isonitrogenous with the addition of appropriate amounts of glycine according to method described by our previous laboratory study (Lin and Zhou 2006). Procedures for diet preparation and storage were the same as described by our laboratory previous study (Lin and Zhou 2006).

Fish and feeding trial

Hatchery-reared juvenile Jian carp were obtained from the Tong-Wei Hatchery (Sichuan, China). Before starting the experiment, the fish were acclimatized to the experimental conditions for 4 weeks. A total of 300 fish with an initial weight of 5.36 ± 0.02 g were randomly assigned to each of 6 experimental aquaria (90 L × 30 W × 40 H cm). Each aquaria was connected to a closed recirculating water system and an oxygen auto-supplemented system. The water flow rate in each aquarium was maintained at 1.2 L min^{-1}, and the water was drained through biofilters to remove solid substances and reduce the ammonia concentration. The water temperature and pH value were 23 ± 1 °C and 7.0 ± 0.3, respectively. The control and glutamine groups were fed basal and glutamine-supplemented diets, respectively; and the basal and glutamine-supplemented diets were randomly assigned to triplicate aquaria. For the feeding trial, each of the diets was fed to a triplicate of fish six times per day for the first 4 weeks and four times per day from the 5th to 6th week, a feeding rhythm that was established in previous study (Kuang et al. 2012). Thirty minutes after the feeding, uneaten feed was removed by siphoning and then air-dried. Total number and body weight of fish in each aquarium were measured at the beginning and the end of the feeding trial.

Sample collection

The procedures of serum and tissue sample collection were similar to previously described by Kuang et al. (2012). Prior to sampling, the fish were starved for 12 h according to Chong et al. (2002) and then anaesthetized with benzocaine bath (50 mg L^{-1}) as described by Berdikova et al. (2007). Immediately,

Table 1 Formulation and nutrient content of the basal diet

Ingredients (g kg^{-1} diet)		Nutrient content (g kg^{-1} diet)[a]	
Fish meal	125.0	Crude protein	338.4
Gelatin	100.0	Crude lipid	45.6
Casein	180.0	Crude ash	61.8
α-starch	150.0	Available phosphorus	6.0
Corn starch	293.2	Lysine	21.9
Soybean oil	18.0	Methionine + cystine	14.4
Fish oil	18.0	n-3 fatty acids	10.0
$Ca(H_2PO_4)_2$	24.0	n-6 fatty acids	10.0
Amino acid premix[b]	50.0		
Cellulose	20.0		
Choline chloride (500 g kg^{-1})	1.3		
Trace mineral premix[c]	10.0		
Vitamin premix[d]	10.0		
Ethoxyquin (300 g kg^{-1})	0.5		
Total	1000.0		

[a] Crude protein, crude lipid, and crude ash content were determined according to the method of AOAC (1998). Available phosphorus, lysine, Methionine + cystine, n-3 and n-6 fatty acid contents were calculated according to NRC (1993)

[b] Glutamine was added to obtain glutamine premix. The amino acid mixture from basal diet was made isonitrogenous with addition of reduced amounts of glycine and compensated with appropriate amounts of corn starch. Per kilogram of amino acid premix composition from basal and glutamine supplementation diet was as follows (g kg^{-1}): glutamine 0, 242.42 g, DL-methionine 111.11, 111.11 g, L-threonine 105.58, 105.58 g, L-glycine 249.29, 0 g, respectively. 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 g kg^{-1} copper) 1.20 g, $MnSO_4.H_2O$ (318 g kg^{-1} manganese) 4.09 g, KI (38 g kg^{-1} iodine) 2.90 g, $NaSeO_3$ (10 g kg^{-1} selenium) 2.50 g, $ZnSO_4 \cdot 7H_2O$ (225 g kg^{-1} zinc) 21.64 g, $FeSO_4 \cdot 7H_2O$ (197 g kg^{-1} iron) 69.70 g. All ingredients were diluted with $CaCO_3$ to 1 kg

[d] Per kilogram of vitamin premix (g kg^{-1}): retinyl acetate (500,000 IU g^{-1}) 0.80 g, cholecalciferol (500,000 IU g^{-1}) 0.48 g, DL-α-tocopherol acetate (500 g kg^{-1}) 20.00 g, menadione (500 g kg^{-1}) 0.20 g, cyanocobalamin (100 g kg^{-1}) 0.01 g, D-biotin (200 g kg^{-1}) 0.50 g, folic acid (960 g kg^{-1}) 0.52 g, thiamin nitrate (980 g kg^{-1}) 0.10 g, ascorbyl acetate (920 g kg^{-1}) 7.24 g, niacin (980 g kg^{-1}) 2.85 g, meso-inositol (980 g kg^{-1}) 52.86 g, calcium-D-pantothenate (980 g kg^{-1}) 2.51 g, riboflavin (800 g kg^{-1}) 0.63 g, pyridoxine hydrochloride (980 g kg^{-1}) 0.76 g. All ingredients were diluted with corn starch to 1 kg

blood of 25 fish from each aquarium was drawn from the caudal vein, stored at 4 °C overnight and then centrifuged at 3000×*g* for 10 min at 4 °C to collect serum. The serum was stored at −80 °C until analysis for immune parameters according to Zuo et al. (2012). After blood collection, the head kidney and spleen were removed quickly, weighed, frozen in liquid nitrogen, and then stored at −80 °C until analysis.

Serum immune parameter analysis

The contents of complement 3 (C3) and 4 (C4) were assayed according to the method of Welker et al. (2007). The serum lysozyme activity was assayed as the methods described by El-Boshy et al. (2010).

Analysis of gene expression

The RNA isolation and cDNA synthesis were performed as previously described by our laboratory (Wu et al. 2011). Total RNA of samples were isolated using RNAiso Plus (TaKaRa Biotechnology, Dalian Co. Ltd, China) according to the manufacturer's instructions followed by DNase I treatment. RNA quantity and quality were assessed by electrophoresis on 1 % agarose gels and by spectrophotometry at 260 and 280 nm. cDNA was synthesized using a PrimeScript™ RT reagent Kit (TaKaRa Biotechnology, Dalian Co. Ltd, China) and according to manufacturer's instructions. Specific primers for the target genes (IL-1β, TNF-α, IL-10, and TGF-β2) and housekeeping gene (β-actin) were designed following the published

sequences of common carp and the same as described by our laboratory previous study (Wu et al. 2013) (Table 2). PCR amplification was performed with a CFX96^{TM} Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) using a SYBR® PrimeScript^{TM} RT-PCR Kit II (TaKaRa Biotechnology, Dalian Co. Ltd, China), according to standard protocols. The Pfaffl method (Pfaffl 2001) was used to compare the relative transcript levels.

Western blot analysis

Protein homogenates from spleen and head kidney were prepared similar to described by Yao et al. (2008). Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Spleen or head kidney lysates were subjected to SDS-PAGE and Western blotting using the appropriate antibody according to the method described by Yao et al. (2008). After being washed, the polyvinylidene difluoride membrane was incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and signals were detected by enhanced chemiluminescence. The antibody selection was according to the method described by Seiliez et al. (2008). Briefly, alignment of amino acid sequences of peptides was used to produce polyclonal antibodies with the corresponding carp sequences to check the specificity of the antibodies; then, preliminary experiments were performed with murine samples as a control to comparative Western blots of carp and murine samples. Thus, the anti-phospho-mTOR (Ser^{2448}) was purchased from Cell Signaling Technologies (Danvers, MA, USA), anti-mTOR and anti-actin were obtained from Abcam (Cambridgeshire, UK), which was checked and successfully cross-reacted with Jian carp proteins of interest. The signals of Western blot were quantitatively measured using the image analysis software Quantity One, v4.62 (Bio-Rad, Hercules, CA, USA).

Antioxidant-related parameter analysis

Sample preparation

The sample preparation for determination of antioxidant parameters was performed as described by Kondoh et al. (2003). Samples of the spleen and head kidney were each homogenized in 10 volumes (w/v) of ice-cold physiological saline solution and centrifuged at 6000×*g* for 20 min at 4 °C, respectively. After centrifugation, the supernatant was used for determination of antioxidant parameters.

Oxidative damage analysis

Lipid peroxidation was analyzed as described by Livingstone et al. (1990) and measured in terms of malondialdehyde (MDA) equivalents using the thiobarbituric acid (TBA) reaction. The spleen and head kidney protein carbonyl content were determined according to the method described by Baltacioğlu et al. (2008), with a minor modification using the 2,4-

Table 2 Primers used in this study

Gene	Accession No.	Primer sequence		Thermocycling conditions
IL-1β	AB010701	F	5′-AAC TTC ACA CTT GAG GAT-3′	95 °C 30 s, 40 cycles of 95 °C 5 s, 61.5 °C 30 s and 72 °C 30 s
		R	5′-GAC AGA ACA ATA ACA ACA AC-3′	
TNF-α	AJ311800	F	5′-AAG TCT CAG AAC AAT CAG GAA-3′	95 °C 30 s, 40 cycles of 95 °C 5 s, 61.5 °C 30 s and 72 °C 30 s
		R	5′-TGC CTT GGA AGT GAC ATT-3′	
IL-10	AB110780	F	5′-GCC AGC ATA AAG AAC TCG-3′	95 °C 30 s, 40 cycles of 95 °C 5 s, 59.4 °C 30 s and 72 °C 30 s
		R	5′-CCA AAT ACT GCT CGA TGT-3′	
TGF-β2	CCU66874	F	5′-GGG ACA TCA TCG CCA TCT-3′	95 °C 30 s, 40 cycles of 95 °C 5 s, 57.5 °C 30 s and 72 °C 30 s
		R	5′-TGA CAT TCT CGG CAG GGT-3′	
β-actin	M24113	F	5′-CGT GAT GGA CTC TGG TGA TG-3′	95 °C 10 s, 40 cycles of 95 °C 5 s, 60 °C 30 s
		R	5′-TCG GCT GTG GTG GTG AAG-3′	

F forward, *R* reverse

dinitrophenylhydrazine (DNPH) reagent. The protein carbonyl content was calculated from the peak absorbance at 340 nm, using an absorption coefficient of 22,000 M^{-1} cm^{-1}.

Detection of antioxidant enzyme activities and GSH content

The anti-superoxide anion (ASA) capacity ($O_2^{\cdot-}$-scavenging ability) and the anti-hydroxyl radical (AHR) capacity ($^{\cdot}OH$-scavenging ability) were determined by the method described by Jiang et al. (2009). Briefly, superoxide radicals were generated by the action of xanthine and xanthine oxidase. With the addition of an electron acceptor, a coloration reaction (absorbance at 550 nm) was developed using the gross reagent. Vitamin C was used as the standard agent. One unit (U) of ASA equated for inhibition of the superoxide anion production by 1 mg vitamin C in this condition.; the AHR was assayed based on the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$). A coloration reaction (absorbance at 550 nm) is also developed using the gross reagent. One unit (U) of AHR equated for the amount that reduced the level of hydrogen peroxide (H_2O_2) by 1 mmol L^{-1} min^{-1}.

Superoxide dismutase (SOD) activity was assayed according to Jin et al. (2013). Glutathione peroxidase (GPx) activity was determined as described by Zhang et al. (2008). Catalase (CAT) activity was determined by the decomposition of hydrogen peroxide by the method of Aebi (1984). Glutathione reductase (GR) activity was measured as described by Lora et al. (2004). Reduced GSH was measured according to the method by Beutler et al. (1963).

Statistical analysis

Data on percent weight gain (PWG), specific growth rate (SGR), feed efficiency (FE), spleen index (SI), head kidney index (HKI), spleen protein content (SPC), and head kidney protein content (HKPC) were calculated:

$$\mathrm{PWG} = \text{weight gain} \times 100/\text{initial weight}$$

$$\mathrm{SGR} = 100 \times (\ln \text{final weigh} - \ln \text{initial weight}) / \text{number of days}$$

$$\mathrm{FE} = 100 \times (\text{weight gain/feed intake})$$

$$\mathrm{SI} = 100 \times (\text{wet spleen weight/wet body weight})$$

$$\mathrm{HKI} = 100 \times (\text{wet head kidney weight/wet body weight})$$

$$\mathrm{SPC} = 100 \times (\text{spleen protein/wet spleen weight})$$

$$\mathrm{HKPC} = 100 \times (\text{head kidney protein/wet head kidney weight})$$

Experimental data were expressed as the mean ± standard deviation (SD). Data were analyzed using *t* test. Statistical analysis was performed using PASW Statistics 18 (IBM, Chicago, IL, USA). $P < 0.05$ was considered significantly different.

Results

Growth performance and development of spleen and head kidney

Dietary glutamine supplementation for 6 weeks significantly increased the final body weight (FBW), PWG, SGR, and SPC ($P < 0.05$), and feed intake (FI, $P < 0.01$) of juvenile Jian carp when compared with the control group (Table 3). The FE in the glutamine group was higher than that in the control group ($P = 0.058$, Table 3). The dietary glutamine supplementation did not significantly influence the survival rate, SI, HKI, and HKPC of juvenile Jian carp ($P > 0.05$, Table 3).

Serum C3 and C4 contents, and lysozyme activity

Effects of dietary glutamine supplementation on serum C3 and C4 contents, and lysozyme activity of fish are presented in Table 4. The levels of serum C3 and lysozyme activity of juvenile Jian carp in the glutamine group were significantly higher than those in the control group ($P < 0.05$). There were no significant differences in the levels of serum C4 between the glutamine and control groups ($P > 0.05$).

Expression of cytokine genes

Effects of dietary glutamine supplement on the expression of cytokine genes in the spleen and head

Table 3 Survival rate (%), initial body weight (IBW, g $fish^{-1}$), final body weight (FBW, g $fish^{-1}$), percent weight gain (PWG, %), specific growth rate (SGR), feed intake (FI, g $fish^{-1}$), feed efficiency (FE, %), spleen index (SI, %), head kidney index (HKI, %) and protein content of spleen (SPC, % of tissue), and head kidney (HKPC, % of tissue) from juvenile Jian carp (*Cyprinus carpio* var. Jian) fed basal (Control) and glutamine-supplemented (12.0 g glutamine kg^{-1} diet, +Glutamine) diets for 6 weeks

	Control	+Glutamine	*P* value
Survival rate	100.0 ± 0.0	100.0 ± 0.0	–
IBW	5.37 ± 0.04	5.35 ± 0.02	0.644
FBW	28.68 ± 0.92	31.29 ± 0.41*	0.011
PWG	434.50 ± 20.96	484.31 ± 10.05*	0.021
SGR	3.99 ± 0.09	4.20 ± 0.04*	0.022
FI	30.21 ± 0.05	32.17 ± 0.29**	<0.001
FE	87.69 ± 3.50	93.18 ± 0.88	0.058
SI	2.95 ± 0.40	2.91 ± 0.65	0.745
HKI	3.66 ± 0.64	3.85 ± 0.64	0.237
SPC	16.95 ± 0.76	23.65 ± 3.94*	0.045
HKPC	17.27 ± 1.79	18.08 ± 1.90	0.502

Data represent mean ± SD

* Significant difference between the two diets ($P < 0.05$)

** Significant difference between the two diets ($P < 0.01$)

Table 4 The levels of complement 3 (C3, mg L^{-1}), complement 4 (C4, mg L^{-1}) and lysozyme activity (U mL^{-1}) in serum from juvenile Jian carp (*Cyprinus carpio* var. Jian) fed basal (Control) and glutamine-supplemented (12.0 g glutamine kg^{-1} diet, +Glutamine) diets

	Control	+Glutamine	*P* value
C3	66.52 ± 9.29	78.25 ± 5.61*	0.042
C4	19.42 ± 2.71	20.50 ± 2.44	0.528
Lysozyme	2.30 ± 0.25	2.74 ± 0.29*	0.035

Data represent mean ± SD of three replicate groups, with five fish in each group

* Significant difference between the two diets ($P < 0.05$)

kidney of juvenile Jian carp are presented in Fig. 1. In the spleen, the levels of IL-1β (Fig. 1a) and IL-10 (Fig. 1c) mRNA were significantly lower in the glutamine group than that in the control group ($P < 0.01$), but glutamine did not change the levels of TNF-α (Fig. 1b) and TGF-β2 (Fig. 1d) mRNA ($P > 0.05$). In the head kidney, significantly lower levels of TNF-α (Fig. 1b) and IL-10 (Fig. 1c) mRNA, and higher level of TGF-β2 mRNA (Fig. 1d) were found in the glutamine group as compared to the control group ($P < 0.05$). However, no statistical difference was observed in the IL-1β mRNA level in the head kidney between the two dietary groups ($P > 0.05$, Fig. 1a).

TOR phosphorylation

Using Western blot analyses, we investigated the effects of glutamine on phosphorylation of TOR (p-TOR) in the spleen and head kidney of juvenile Jian carp. Phosphorylation of TOR on residue Ser^{2448} has been used to monitor the activation of TOR (Weichhart et al. 2008). As shown in Fig. 2, level of p-TOR in the spleen was enhanced in fish fed glutamine-supplemented diet ($P < 0.01$). Since both groups showed equal abundance of total TOR ($P > 0.05$), the p-TOR to total TOR protein ratio was increased in the spleen of fish fed glutamine-supplemented diet ($P < 0.01$). In the head kidney, although similar quantities of proteins were loaded on the gels, the p-TOR ($P < 0.01$) and TOR ($P = 0.062$) protein levels were higher in fish fed basal diet compared with that in fish fed glutamine-supplemented diet (Fig. 2). Consequently, the ratio between p-TOR and total TOR protein was not different in fish fed basal and glutamine-supplemented diets ($P > 0.05$, Fig. 2). This result suggested that the synthesis of TOR protein may be required to restore TOR activity in the head kidney of fish fed basal diet.

Antioxidant-related parameters

As shown in Fig. 3, the MDA contents and ASA capacity in the spleen and head kidney of juvenile Jian carp, as well as the protein carbonyl contents and AHR capacity in the head kidney of fish, showed no significant differences between the two dietary groups ($P > 0.05$). The glutamine group showed lower protein carbonyl ($P = 0.065$) and significantly higher AHR capacity in the spleen of fish ($P < 0.01$).

The activities of SOD, CAT, GPx, and GR, as well as GSH content in the spleen and head kidney of juvenile Jian carp are presented in Table 5. The SOD, CAT, GPx, and GR activities in the spleen and head kidney, and GSH content in the head kidney were not statistically different between the two dietary groups ($P > 0.05$). The GSH content in the spleen was

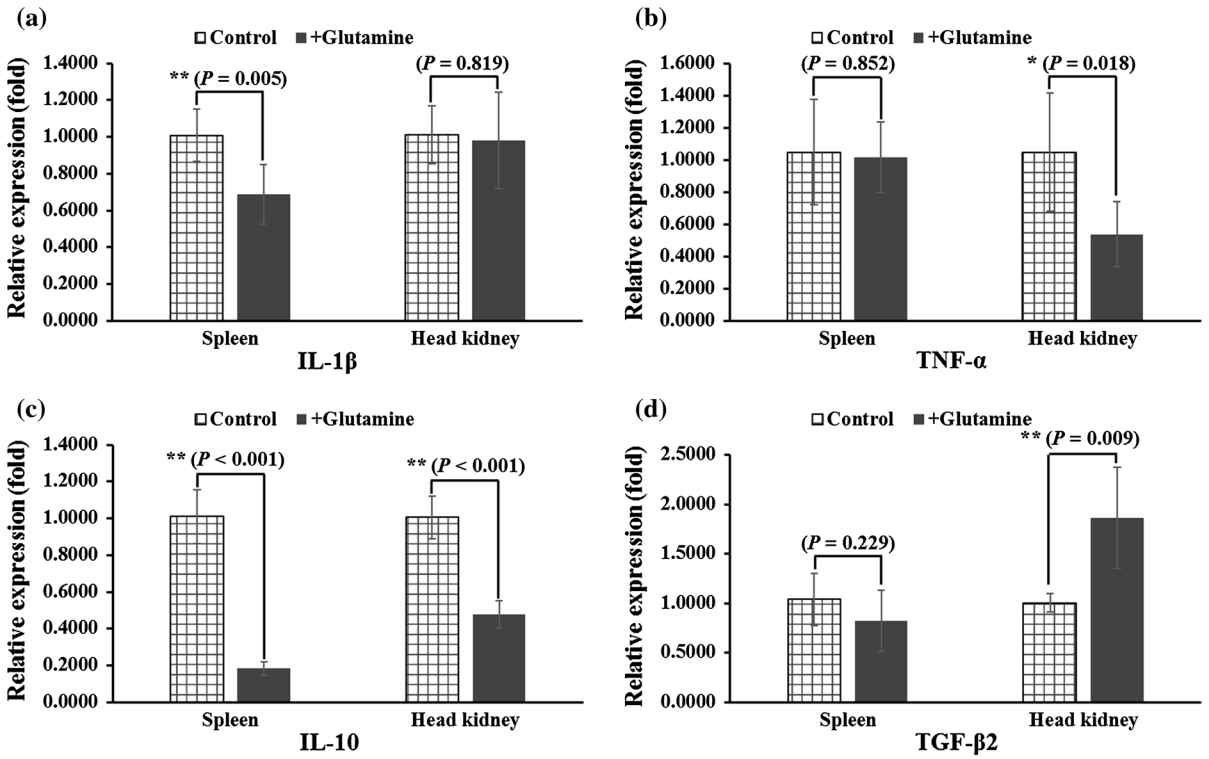

Fig. 1 Relative gene expression of interleukin 1β (IL-1β, **a**), tumor necrosis factor α (TNF-α, **b**), interleukin 10 (IL-10, **c**) and transforming growth factor β2 (TGF-β2, **d**) in the spleen and head kidney from juvenile Jian carp (*Cyprinus carpio* var. Jian) fed basal (Control) and glutamine-supplemented (12.0 g glutamine kg^{-1} diet, +Glutamine) diets. Data represent mean ± SD of three replicate groups, with 6 fish in each group. *Asterisk* denotes significant difference between the two diets ($P < 0.05$); and *double asterisks* denote significant difference between the two diets ($P < 0.01$)

significantly increased in fish fed glutamine-supplemented diet when compared with fish fed the basal diet ($P < 0.01$).

Discussion

Effects of dietary glutamine supplementation on the growth performance, serum immunity parameters, and growth of spleen and head kidney of fish

In the present study, supplementation of 12.0 g glutamine kg^{-1} diet significantly enhanced the growth performance of juvenile Jian carp, which was similar to previous study from our laboratory (Lin and Zhou 2006), and hybrid striped bass (Cheng et al. 2012). Growth of fish partly depends on the fish health which is related to the immune competence (Pohlenz et al. 2012). Lysozyme, and C3 and C4 contents are important defense molecule of the innate immune system in fish (Jin et al. 2013). Our data showed that dietary glutamine enhanced the lysozyme activity and C3 contents in the serum of Jian carp. Similarly, dietary glutamine supplementation has been shown to increase serum lysozyme activity in hybrid striped bass (Cheng et al. 2012) and C3 levels in juvenile hybrid sturgeon (Zhu et al. 2011). These data suggested that glutamine could enhance the innate immunity of fish.

The spleen and head kidney are important immune organs of fish (Rauta et al. 2012; Rønneseth et al. 2007). In the present study, the SI and HKI of juvenile Jian carp were not changed by dietary glutamine supplementation. Similarly, the relative size of spleen and head kidney was not affected by dietary glutamine supplementation in channel catfish (Pohlenz et al. 2012). The reasons for these results are still unclear, but may be explained by the preferred protection of critical organs when fish faces malnutrition.

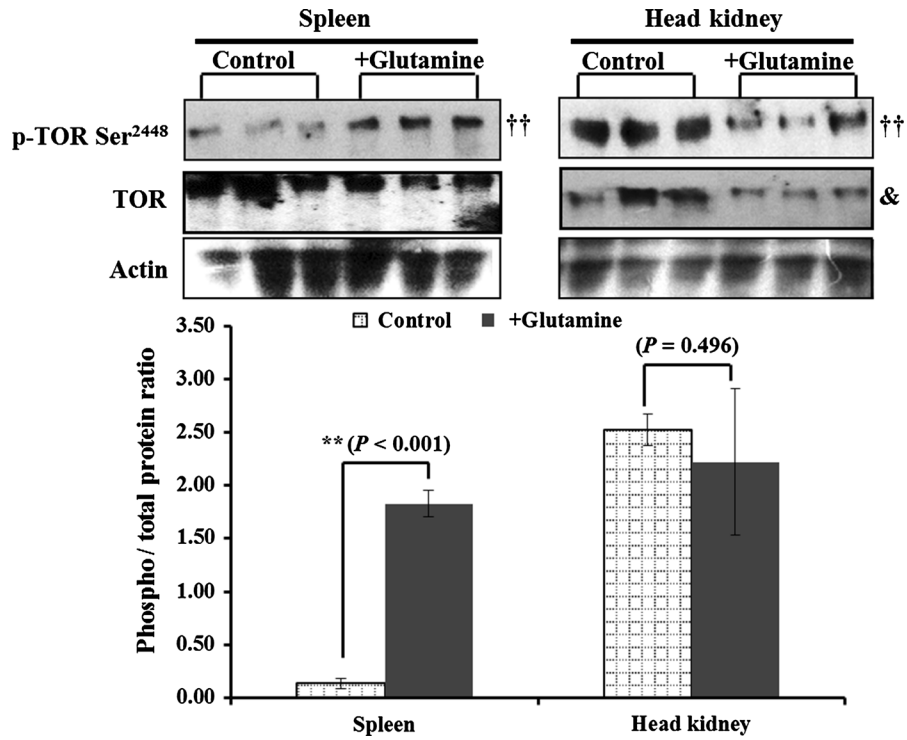

Fig. 2 Western blot analysis of TOR protein phosphorylation at Ser^{2448} in the spleen and head kidney from juvenile Jian carp (*Cyprinus carpio* var. Jian) fed basal (Control) and glutamine-supplemented (12.0 g glutamine kg^{-1} diet, +Glutamine) diets. A representative blot is shown. *Data* represent mean ± SD of three replicate groups, with six fish in each group. *Double dagger* (††) denote significant difference in the phosphorylation of TOR (p-TOR) between the two diets ($P < 0.01$); *ampersand* (&) denotes difference in the total TOR between the two diets ($P = 0.062$); and *double asterisks* denote significant difference in p-TOR/TOR between the two diets ($P < 0.01$)

Furthermore, our data showed that glutamine increased the spleen protein content, which may partly attribute to its benefit effect on the immunological defense systems in fish. The increased protein content of spleen may partly ascribe to the enhanced protein synthesis by glutamine in fish. To our knowledge, the TOR kinase signaling plays an important role in protein synthesis in vertebrates, and p-TOR on residue Ser^{2448} has been used to monitor the activation of TOR (Weichhart et al. 2008). In this study, glutamine increased the level of p-TOR in the spleen of fish, supporting our hypothesis that glutamine-increased splenic protein content was partly related to the activation of TOR. Unexpectedly, dietary glutamine supplementation did not change the protein contents in the head kidney of juvenile Jian carp, whereas unsupplemented with glutamine induced increases of the p-TOR and total TOR protein levels in this organ. To date, studies have not addressed the effects of glutamine on the TOR in the head kidney of fish. The reasons for those results are still unknown, but may be explained by the adaptive mechanism that increasing of p-TOR and total TOR protein levels contributes to ensuring the protein of head kidney when glutamine is absent. A review indicated that glutamine deprivation also induced mTOR activation to inhibit autophagic processes, promoting cell survival during physiological stress in rat small intestinal epithelial (Bertrand et al. 2012). This reference supported our hypothesis.

Effects of dietary glutamine supplementation on cytokines in the spleen and head kidney of fish

Fish live in a water environment where they are constantly exposed to various pathogens and stress factors which induce an inflammatory response that lead to tissue injury (Saurabh and Sahoo 2008; Troncoso et al. 2012). Pro-inflammatory cytokines such as IL-1β and TNF-α are indicated to mediate the inflammatory response in fish (Reyes-Cerpa et al. 2013). Wischmeyer et al. (2001) reported that the pro-inflammatory cytokines must be tightly controlled to avoid organ damage to the rats. Like a terrestrial animal, the production of pro-inflammatory cytokines was counteracted by anti-inflammatory cytokines including IL-10 and TGF-β in fish (Reyes-Cerpa et al. 2013). In the present study, dietary glutamine supplementation down-regulated the IL-1β and IL-10 gene expressions, but did not affect the levels of TNF-α and TGF-β2 mRNA in the spleen of fish. To our

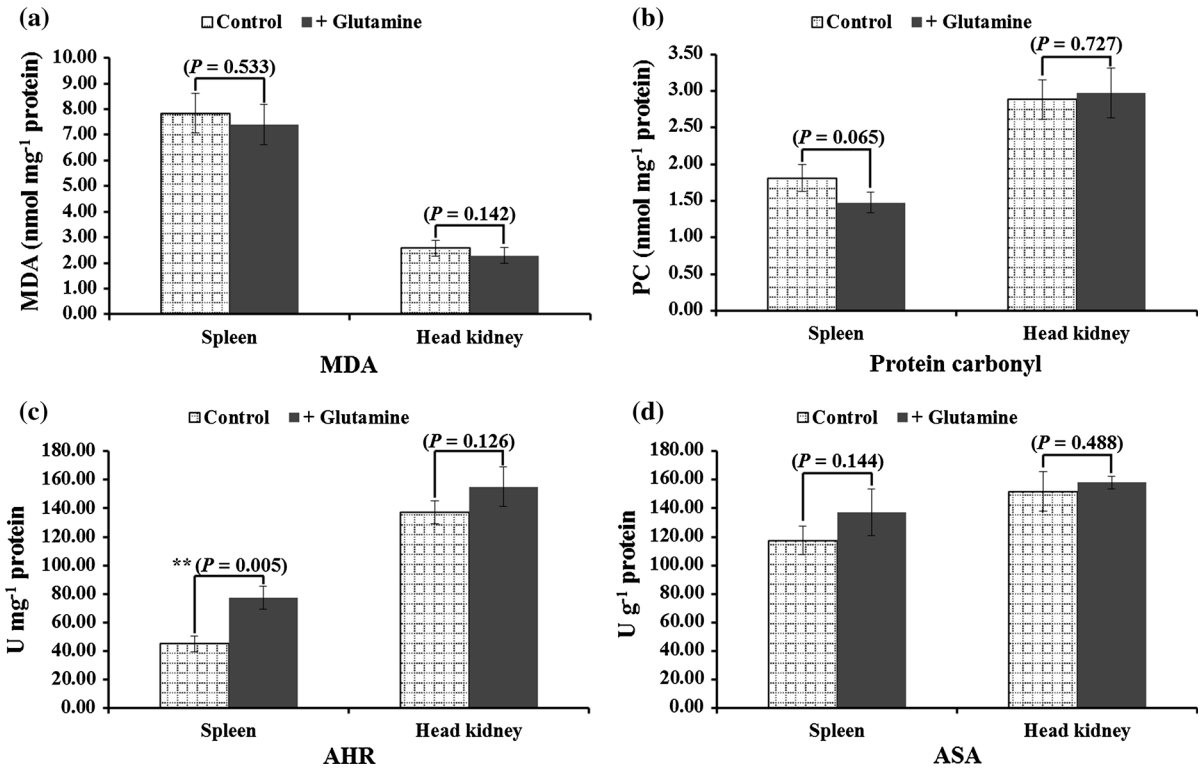

Fig. 3 Lipid peroxidation (expressed as MDA formation, nmol mg^{-1} protein, **a**), protein oxidation (expressed as protein carbonyl, nmol mg^{-1} protein, **b**), anti-hydroxyl radical (AHR, U mg^{-1} protein capacity, **c**) and anti-superoxide anion (ASA, U g^{-1} protein capacity, **d**) in the spleen and head kidney from juvenile Jian carp (*Cyprinus carpio* var. Jian) fed basal (Control) and glutamine-supplemented (12.0 g glutamine kg^{-1} diet, +Glutamine) diets. *Data* represent mean ± SD of three replicate groups, with six fish in each group. *Double asterisks* denote significantly different between the two diets ($P < 0.01$)

knowledge, no information is available concerning the effects of glutamine on cytokines in fish, but similar patterns for the IL-1 in jejunum of piglets (Zhong et al. 2012) and IL-10 levels in intestines of rats (Ding and Li 2003) were observed. These results imply that glutamine down-regulated the pro-inflammatory cytokine IL-1β gene expression, which may contribute to avoiding damage to the spleen of fish. In addition, glutamine decreased the level of IL-10 mRNA, which might be due to the reduction in IL-1β in the spleen of fish, since Souza et al. (2003) reported that IL-10 production could be regulated by IL-1 in the intestine of rats. However, the detailed mechanism of these results needs further investigation.

To our knowledge, the expression of cytokines is regulated by intracellular signaling molecule in terrestrial animal. It was reported that inhibited mTOR promoted production of pro-inflammatory cytokines in human immune cells (Weichhart et al. 2008). This study observed that fish fed glutamine supplementation diets increased the level of p-TOR in the spleen of fish, suggesting that glutamine down-regulated pro-inflammatory cytokines IL-1β, which may be partially mediated by TOR kinase signaling. However, the underlying mechanisms need to be further investigated.

The current study observed that diet unsupplemented with glutamine caused increase of the levels of TNF-α and IL-10 mRNA in the head kidney of juvenile Jian carp. The up-regulation of anti-inflammation cytokine IL-10 gene expression in the head kidney of fish fed basal diet could be an adaptation to the high levels of pro-inflammation cytokine TNF-α in the current study. However, the p-TOR and total TOR were also induced when glutamine was absent in the head kidney of juvenile Jian carp. As mentioned above, mTOR can inhibit pro-inflammation cytokines in human immune cells (Weichhart et al. 2008). Thus,

Table 5 The activities of superoxide dismutase (SOD, U mg^{-1} protein), catalase (CAT, U mg^{-1} protein), glutathione peroxidase (GPx, U mg^{-1} protein) and glutathione reductase (GR, U g^{-1} protein), and glutathione (GSH, mg g^{-1} protein) contents in the spleen and head kidney from juvenile Jian carp (*Cyprinus carpio* var. Jian) fed basal (Control) and glutamine-supplemented (12.0 g glutamine kg^{-1} diet, +Glutamine) diets

	Control	+Glutamine	*P* value
SOD			
Spleen	21.31 ± 1.80	24.91 ± 2.94	0.144
Head kidney	28.15 ± 2.58	29.34 ± 0.83	0.488
CAT			
Spleen	2.78 ± 0.43	2.94 ± 0.36	0.502
Head kidney	3.30 ± 0.28	3.69 ± 0.50	0.127
GPx			
Spleen	114.75 ± 11.27	121.05 ± 10.83	0.524
Head kidney	510.30 ± 25.80	504.26 ± 34.67	0.821
GR			
Spleen	35.56 ± 5.93	35.41 ± 4.91	0.974
Head kidney	79.54 ± 6.72	81.52 ± 11.57	0.810
GSH			
Spleen	24.35 ± 2.81	47.13 ± 3.77**	< 0.001
Head kidney	34.76 ± 3.60	36.59 ± 4.04	0.591

Data represent mean ± SD of three replicate groups, with 6 fish in each group

** Significantly different between the two diets ($P < 0.01$)

the reasons for the increase of p-TOR may be an adaptive increasing which was used to control excessive pro-inflammation cytokine TNF-a production, but this hypothesis needs further investigation. In addition, this study observed that glutamine supplementation up-regulated the anti-inflammation cytokine TGF-β2 gene expression, which may contribute to protecting head kidney against injury in fish. Though little information is available about the relationship between glutamine and cytokines in the head kidney of fish, a similar pattern for the levels of IL-10 and TGF-β2 mRNA in the head kidney from Jian carp fed diets with supplemental isoleucine were observed in our previous laboratory study (Zhao et al. 2013). In another hand, the increased levels of TGF-β2 mRNA by glutamine in the head kidney rather than in the spleen may be corresponding to different subsets and maturation status of the B cells in these tissues of fish. In humans, it is reported that both resting and activated B cells can express TGF-β mRNA (Kehrl et al. 1986). In teleost, developing B cells mature in the head kidney and then migrate to spleen for activation (Zwollo et al. 2005). In this study, the enhancement of TGF-β2 mRNA level in the head kidney of fish fed glutamine-supplemented diet could possibly be due to the improvement in development of B cells in the head kidney of fish. Recent study indicated that glutamine contributed to the proliferation of B cells in humans (Le et al. 2012). However, the detailed mechanism should be further investigated.

Effects of dietary glutamine supplementation on antioxidant status in the spleen and head kidney of fish

Immunity is tightly correlated with normal structural and function of the organs, which is often associated with their antioxidant status (Tort et al. 2003; Kuang et al. 2012). Fish immune organs contain various immune cells (Rauta et al. 2012), which could produce oxidative radicals (Cheng et al. 2012, 2011). It is known that excessive ROS can cause lipid peroxidation and protein oxidation which leads to structural and functional dysfunction of the fish tissues (Martínez-Álvarez et al. 2005). The MDA and protein carbonyl contents are the most widely used markers of the lipid peroxidation and protein oxidation, respectively (Tkachenko et al. 2014). In the present study, we observed that the protein carbonyl contents in the spleen was lower in fish fed the diet supplemented with glutamine compared to the basal diet ($P = 0.065$), but no significant differences were observed in the lipid peroxidation (MDA contents) of spleen. However, information concerning the relationship between glutamine and lipid peroxidation as well as protein oxidation in the spleen of fish is lacking. In mice, glutamine had no effect on the levels of MDA in the liver (Erbil et al. 2005). Our previous studies found that glutamine could decrease (Chen et al. 2009) and repair (Hu et al. 2014) protein oxidative damage in fish enterocytes. The different effects of glutamine on the lipid peroxidation and protein oxidation in the spleen of fish may be associated with the ·OH-scavenging ability. Previous studies showed that murine spleen B (Zhang et al. 2001) and T (Zhang et al. 2002) lymphocyte could generate ROS including ·OH which could directly modifies amino acid side-chains in protein, resulting in a protein oxidative damage (Stadtman and Levine

2003). Girotti and Thomas (1984) reported that because of the short lifetime of the $^{\cdot}OH$, it may collide infrequently with human erythrocyte membranes and the rate of lipid peroxidation is low. Our data showed that the diet supplemented with glutamine enhanced AHR ability, supporting the hypothesis that glutamine decreased protein oxidation, which may be partially through enhanced intracellular $^{\cdot}OH$-scavenging ability in the spleen of fish. In humans, the GSH, CAT, and GPx are majorly responsible for the clearing $^{\cdot}OH$ (Valko et al. 2007). In the current study, dietary glutamine supplementation increased the GSH content, but did not change the CAT and GPx activities in the spleen of fish. These results suggested that the glutamine-improved $^{\cdot}OH$-scavenging ability may be mainly through the increase of the GSH content (rather than CAT and GPx) in the spleen of fish. GSH content is always influenced by its biosynthesis and regeneration. Glutamine can provide intracellular glutamate as a substrate for GSH biosynthesis in rat gut (Cao et al. 1998). This gives a potent that the glutamine supplementation may increase the biosynthesis of GSH. In addition, GR has been recognized to be involved in electron transfer and GSH regeneration by reducing the GSSG in terrestrial animals (Lillig et al. 2008). In the present study, glutamine did not affect the GR activity in the spleen, suggesting that the glutamine affects the GSH content did not by GR in the spleen of fish. Furthermore, $O_2^{\cdot-}$ also as a species of oxygen radicals can cause a wide range of oxidative damage within the cell (Bai and Cederbaum 2001). However, the data in the current study showed that dietary glutamine supplementation did not affect the ASA ability in the spleen of fish. This result suggested that the prevention of protein oxidation by glutamine may be not involving the ASA in the spleen of fish. SOD is the important enzyme to respond against $O_2^{\cdot-}$ (Valko et al. 2007). In the present study, SOD activity in the spleen was not affected by dietary glutamine supplementation, which might partly explain why the glutamine supplementation in the diet did not affect the ASA in the spleen of fish. In a word, our results suggested that glutamine-inhibited protein oxidation, which may be partly through the enhanced $^{\cdot}OH$-scavenging ability via elevating GSH content in the spleen of fish.

In contrast, in the head kidney of fish, the MDA, protein carbonyls, as well as SOD, CAT, GPx, and GR activities were not affected by glutamine, suggesting that glutamine did not affect antioxidant status in the head kidney of fish. Meanwhile, glutamine supplementation also has no effect on the GSH content in the head kidney, whereas it increased the GSH content in the spleen of fish. Similarly, glutamine did not affect the level of renal GSH in rats (Mora et al. 2003). The reason for this difference was possibility that the head kidney contains higher levels of GSH than spleen in the control group (head kidney is 1.4-fold higher than spleen), which might be enough to resist oxidative radicals. In addition, the different patterns between the spleen and head kidney by glutamine in the present experiment may be partially due to the differences of immune cell populations from the spleen and head kidney of fish. Previous studies on the immune cells in common carp (*C. carpio* L.) have revealed a difference in B lymphocyte subpopulations in the spleen compared with head kidney (Koumans-van et al. 1995). However, the detailed mechanism should be further investigated.

Conclusion

In summary, results from the present study showed that dietary supplementation with glutamine improved the growth and serum immune parameters in Jian carp. In addition, our findings firstly observed differential effects of glutamine on the regulation of gene expression of cytokines, TOR kinase activity, and antioxidant statues between the spleen and head kidney of fish. In other words, in the spleen, glutamine could down-regulate the pro-inflammatory cytokine IL-1β gene expression, which may be ascribed to increase of TOR signaling. Furthermore, it inhibited protein oxidation, which at least in part may be through the enhanced $^{\cdot}OH$-scavenging ability via elevating GSH content in the spleen of fish. However, in the head kidney, glutamine up-regulated the anti-inflammatory cytokine TGF-β2 gene expression, but down-regulated the pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine IL-10 gene expressions as well as p-TOR and total TOR protein levels. Moreover, the antioxidant-related parameters were not affected by glutamine in the head kidney of fish. Further research is still necessary to elucidate the glutamine metabolism as well as the role of its metabolites in the spleen and head kidney of fish.

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