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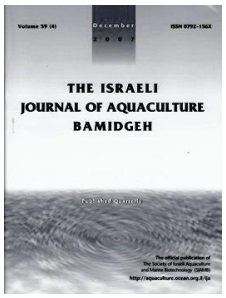

Effect of Dietary Vitamin C on Growth Performance, Antioxidant Status and Innate Immunity of Juvenile Pufferfish (*Takifugu obscurus*)

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Keywords: *Takifugu obscurus*; vitamin C; growth performance; antioxidant status; immune response

Abstract

Six practical diets were formulated to contain 2.6 (the basal diet), 48.9, 95.5, 189.83, 382.4, 779.53 mg/kg vitamin C diet. After 8 weeks of feeding, growth performance, antioxidant status, and innate immunity of juvenile pufferfish were analyzed. Results showed that specific growth rate and protein efficiency ratio in fish fed diets supplemented with vitamin C were significantly higher than in fish fed the control diet. Dietary vitamin C significantly up-regulated the expression levels of growth hormone receptors and Insulin-like growth factor. Blood cell numbers and respiratory burst activity were enhanced in fish fed vitamin C diet. Serum alkaline phosphatase, glutamic-pyruvic transaminase, and glutamic-oxaloacetic transaminase activity were significantly influenced by the dietary vitamin C levels. Fish fed diets supplemented with vitamin C showed increased hepatic superoxide dismutase, glutathione peroxidase, and catalase activity, but decreased malondialdehyde activity. In addition, we found that dietary vitamin C can significantly up-regulate the expression levels of immune-related genes (HSP70, HSP90, BAFF, TNF-α and IL-6) in liver. These results indicate that dietary vitamin C can significantly influence growth performance, antioxidant status, and innate immunity of pufferfish.

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Introduction

Vitamins are low molecular weight organic compounds, which are essential micronutrients for normal growth, reproduction, and health. In aquatic animals, vitamins are obtained mainly through the diet because of either a lack of key enzymes involved in their synthesis, or failure to produce them in sufficient amounts (Drouin et al., 2011; Vélez-Alavez et al., 2014). Vitamin C, known as ascorbic acid, is a water-soluble vitamin that acts as a co-substrate for hydroxylase and oxygenase enzymes involved in the biosynthesis of red blood cells, collagen, carnitine, and neurotransmitters (Tolbert, 1979; Dariasa et al., 2011). Vitamin C is also a powerful antioxidant, which leads to rapid wound healing (Pagador and Din, 2001). It plays a key role in growth, reproduction, the immune system, and response to stressors (Ming et al., 2012; Wan et al., 2014). However, fish cannot synthesize vitamin C due to the lack of L-gulonolactone oxidase enzyme which is the key enzyme for vitamin C synthesis (Fracalossi et al., 2011). Therefore, an exogenous source of vitamin C is required in fish diets. Inadequate supply of dietary vitamin C in fish usually leads to a number of symptoms such as anorexia, retarded growth, spinal deformation, and depressed immunity, which cause significant losses in practical fish farming, especially during the sensitive early stages (Wan et al., 2014; Halver et al., 1969).

Vitamin C requirements have been determined for several cultured fish species, such as yellow croaker (Ai et al., 2006), parrot fish (Wang et al., 2003), Nile tilapia (Ibrahem et al., 2010), cobia (Zhou et al., 2012), Wuchang bream (Wan et al., 2014) and largemouth bass (Chen et al., 2015). The quantitative requirements of vitamin C have been estimated for several species and the recommended values varied in various studies (NRC, 2011). Dietary vitamin C requirement is affected by fish species, fish size, fish age, various environmental factors, and nutrient interrelationships (Ai et al., 2006).

River pufferfish (*Takifugu obscurus*), widely distributed in the Sea of Japan, the East China Sea, and the Yellow Sea, is an anadromous and commercially important fish. Wild resources of pufferfish have sharply decreased due to water pollution and overfishing. With the success of artificial propagation, farmed pufferfish have become increasingly popular in Chinese markets because of their rapid growth and high nutritional quality. The annual production of farmed pufferfish in freshwater has reached an annual level of 20,000–30,000 metric tons. Currently, several nutrition researchers have focused on macronutrient requirements in this species (Yang and Chen, 2013; Liu et al., 2014). However, information regarding the nutritional micronutrient requirement such as vitamin C in pufferfish is still not available. Therefore, the present study was undertaken to evaluate the effects of vitamin C on growth, some hematological parameters, nonspecific immunological parameters, growth related gene expression (GHR and IGF-1), and immune related gene expression (BAFF, TNF-α, IL-6, HSP70 and HSP90) in pufferfish.

Materials and methods

The formulation and proximate composition of the basal diet shown in Table 1. The ingredients were purchased from the institute of animal science, Guangdong academy of agriculture science (Guangdong, China). The L-ascorbyl-2-monpolyphosphate (35% ascorbic acid equivalent, DSM, Netherland) was selected as the source of vitamin C. Basal diets were supplemented with six vitamin C levels (0, 50, 100, 200, 400 and 800 mg/kg diet) at the expense of small amounts of cellulose. A basal diet without vitamin C served as the control diet. The final vitamin C concentrations in the six diets were 2.6, 48.9, 95.5, 189.83, 382.4, 779.53 mg/kg respectively, as determined by high-performance liquid chromatography (HPLC). All ingredients were ground into fine power through a 60 mesh. They were thoroughly mixed until homogenous in a Hobart-type mixer, then lipid and water were added and thoroughly mixed. Each mixture was pelleted (2 mm diameter) using a laboratory pellet machine (Institute of Chemical Engineering, South China University of Technology, Guangzhou, China). After air-drying, all samples were sealed in plastic bags, and stored frozen (-20°C).

Table 1. Formulation of the basal diet.

Ingredients	*%*
Fish meal	38.00
Soybean meal	18.00
Peanut meal	16.00
Yeast meal	2.00
Mixed oil[a]	4.00
Wheat meal	13.50
$Ca(H_2PO_4)_2$	2.00
Choline chloride	0.50
immunopotentiator	2.00
Cellulose	2.00
Mineral premix[b]	1.00
Vitamin premix (vitamin C free) [c]	1.00
Proximate composition	
Crude protein	41.94
Crude lipid	8.94
Ash	6.16

[a]1:1 mixture of fish oil and Soybean oil.
[b] Mineral premix (g/kg of mixture): $MgSO_4 \cdot 7H_2O$, 80.0; $NaH_2PO_4 \cdot 2H_2O$, 370.0; KCl, 130.0; $FeSO_4 \cdot 7H_2O$, 40.0; $ZnSO_4 \cdot 7H_2O$, 20.0; Ca-lactate, 356.5; $CuSO_4$, 0.2; $AlCl_3 \cdot 6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4 \cdot H_2O$, 2.0; $CoCl_2 \cdot 6H_2O$, 1.0.
[c] Vitamin premix (g/kg of mixture): myoinositol, 40.0; niacin, 36.0; DL-α-tocopheryl acetate, 20.0; thiamin hydrochloride, 4.0; riboflavin, 9.0; pyridoxine hydrochloride, 4.0; $_D$-pantothenic acid hemicalcium salt, 14.5; D-biotin, 0.3; folic acid, 0.8; menadione,0.2; retinyl acetate, 1.0; cholecalciferol, 0.05; cyanocobalamin, 0.01.

Pufferfish were obtained from a fish farm in Panyu (Guangdong, China). Prior to the start of the experiment, the experimental fish were acclimatized at laboratory conditions for two weeks. The basal diets were fed to all fish for two weeks. At the beginning of the experiment, 360 fish averaging 10.3 ± 0.21 g (mean ± SD) were weighed and randomly distributed into 18 recirculating water tanks (500 L) with 20 individuals per tank. Each experimental diet was randomly assigned to three tanks. Each tank was provided with a continuous flow of water (3 L/min) and continuous aeration through air stones to maintain dissolved oxygen at or near saturation. Fish were fed twice daily (08:00 h and 17:00 h) at a rate of 4-6% wet body weight. The feeding trial lasted for 8 weeks. During the experimental period, water temperature ranged from 25-28°C, pH was 7.5-7.8, dissolved oxygen was no less than 6.0 mg/L, and ammonia nitrogen was lower than 0.05 mg/L.

At the end of the rearing experiment, fish were fasted for 24 h before sampling. Total number, body weight, body length, and viscera of fish in each tank were measured. Six fish from each tank were anesthetized in diluted MS-222 (tricaine methanesulfonate, Sigma, USA) at a concentration of 100 mg/L, then blood for sampling was taken from the caudal vein using a 2mL medical syringe. One part of the blood sample was placed into tubes to determine total blood cell count and respiratory burst activity. The remaining blood was separated by centrifugation, and the supernatants were removed and stored at -80°C for subsequent analysis. The liver was frozen in liquid nitrogen and stored at -80°C for later analysis.

A drop of diluted blood sample (10 μL) was placed on a hemocytometer to count cells with light microscope (Olympus). To monitor the level of respiratory burst, a cell-permeant probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma) was used as described by Xian et al. (2009). 200 μL blood cells suspension was incubated with 10 μM DCFH-DA for 30 min in the dark at room temperature. Fluorescence of the cell suspensions was then analyzed using flow cytometer (Becton–Dickinson FACSCalibur). Typically, 10,000 cells were analyzed for the two fluorescent signals. Respiratory burst production was expressed as mean fluorescence of DCF.

Blood biochemical parameters were determined according to the methods described by Kikuchi et al. (1994). The levels of alkaline phosphatase (AKP), cholesterol (CHOL), glutamic-pyruvic transaminase (GPT), and glutamic-oxaloacetic transaminase (GOT) were measured in the automatic biochemical analyzer Beckman Cx-4 (Beckman Coulter,

USA) using kits purchased from Shanghai Junshi Biotech Co., Ltd. All assay kits were specially designed for fish detection.

Samples of liver were homogenized in ice-cold phosphate buffer (1:10 dilution, 0.064 M phosphate buffer, pH 6.4). Then the homogenates were centrifuged at 4000 × g for 20 min at 4°C to remove debris, and the resultant supernatants were used directly for enzyme assays. Malondialdehyde (MDA) content was measured using the thiobarbituric acid colorimetry method (Drape et al., 1993). Total superoxide dismutase (SOD) activity was determined following the method of Panchenko et al. (1975). Alkaline phosphatase (AKP) activity was determined according to Engstad et al. (1992). Glutathione peroxidase (GPx) activity was measured according to the procedure of Bell et al. (1985). Catalase (CAT) activity was analyzed according to the method of Aebi (1984). Hepatic protein content was measured using the Folin method with bovine serum albumin (BSA, Nanjing Jiancheng Biological Engineering Research Institute, China) as a standard.

Total RNA was isolated from liver tissue using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions, and then dissolved in DEPC treated water. The quantity of isolated RNA was later determined by measuring absorbance at 260 and 280 nm using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA), and its integrity was tested by electrophoresis in 1.2% agarose gel. Single-stranded cDNA was synthesized from 1μg total RNA using PrimeScript RT reagent Kit With gDNA Eraser (Takara, Dalian, China) following the manufacturer's instructions. The cDNA templates were then stored at -80°C for later analysis.

Specific primers of genes were designed with Primer Premier 5 (Premier Biosoft International, Palo Alto, CA, USA) according to published pufferfish mRNA (Table 2). The β-actin gene was used as a housekeeping gene, which was amplified using β-actin-F and β-actin-R gene-specific primers. Real-time PCR was amplified in an ABI 7500 real-time PCR machine (Applied Biosystems, USA) using SYBR Premix Ex Taq (Takara, Dalian, China) following the manufacturer's recommendations. The reaction mixtures were 20 μL, containing 2 μL cDNA sample, 0.4 μL ROX, 10 μL 2 × SYBR Premix Ex Taq, 0.4 μL each of the 10 mM forward and reverse primers, and 6.8 μL dH_2O. The real-time PCR conditions were as follows: 94°C for 10 min, then 45 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, followed by 10 min at 72°C. All samples were run in triplicate, and each assay was repeated three times. After finishing the program, the threshold cycle (Ct) values were obtained from each sample. Relative gene expression levels were evaluated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 2. The sequences of primers in this experiment.

Target gene	*Primer sequence (5´-3´)*
GHR	F: ACCTTGTGAAAAGCACCTCTC R: GACAGGTTCCAAAGTCAG
IGF-1	F: CGACACGCTGCAGTTTG R: TGATACAGGTCTATTTCCA
BAFF	F: CCTTCCTCTCAGCAGTGTCC R: CCGCCTCAAAGACAGAAAAG
TNF-α	F: TCGTGGTGGTCCTCTGTTGC R: CTTGGCTTTGCTGCTGATGC
IL-6	F: GCTGGAAAACAAGGTGAGGGA R: TGTGGAAGGTGTCGGGGTAGT
HSP90	F:TTTGGTGTGGGATTTTACTCAGCCTAC R:TTGTCCGTCCTGACTGTAAATGAACCT
HSP70	F:GCAGAAGCCTACCTCGGAAAGAC R:CGCCAAGATCAAAAATCAACACG
β-Actin	F:CATCACCATCGGCAACGAGAGG R:CGTCGCACTTCATGATGCTGTTG

Crude protein, lipid, and ash contents in diets and whole body were all determined by the standard procedures. Crude protein (N × 6.25) was determined according to the Kjeldahl method after acid digestion using an Auto Kjeldahl System (1030- Auto-analyzer, Tecator, Hoganos, Sweden). Crude lipid was evaluated by the ether-extraction method using a Soxtec System HT (Soxtec System HT6, Tecator, Sweden). Ash content was examined by combustion in a muffle furnace at 560°C for 5 h. Moisture was determined by oven drying at 105°C for 6 h. Tissue vitamin C concentration was determined by HPLC (Dionex, Softron, USA).

All data is expressed as means ± standard deviation. Significant differences were evaluated by a one-way ANOVA followed by Duncan's multiple range tests. Statistical analysis was performed using SPSS 18.0 software (SPSS, Chicago, IL, USA). P value $P<0.05$ was statistically significant.

Results

Survival rate (SR) ranged from 94.47%-98.47%, but no significant difference was found among dietary treatments ($P>0.05$). Weight gain rate (WGR) and protein efficiency ratio in fish (PER) significantly increased with the supplementation of vitamin C (Table 3). Compared to the control group, feed conversion ratio (FCR) significantly improved with dietary vitamin C concentrations. Hepatosomatic index (HSI) was significantly affected ($P<0.05$) only by the diets supplemented with 189.83 and 382.40 mg/kg vitamin C.

Table 3. Effect of different dietary levels of vitamin C on growth performance of pufferfish

Dietary vitamin C levels (mg/kg)	*2.6*	*48.9*	*95.5*	*189.83*	*382.4*	*779.53*
SR (%)[1]	98.47±0.77	97±1.96	97.77±2.23	96.7±3.3	97±1.96	94.47±2.91
WGR (%)[2]	143.19±4.95^{a}	178.34±3.36^{b}	173.18±4.18^{b}	172.66±6.24^{b}	179.15±4.04^{b}	175.17±3.68^{b}
FCR[3]	1.89±0.03^{b}	1.54±0.02^{a}	1.59±0.04^{a}	1.62±0.01^{a}	1.63±0.04^{a}	1.67±0.03^{a}
PER(%)[4]	126.78±1.92^{a}	155.05±1.48^{b}	150.36±3.59^{b}	147.21±3.51^{b}	146.64±1.46^{b}	143.22±3.61^{b}
HSI (%)[5]	11.42±0.97^{b}	11.32±0.68^{b}	10.66±0.47^{ab}	8.25±0.93^{a}	8.81±0.86^{a}	12.22±1.16^{b}

Data are means ± SE (n = 9). Means in the same row with different superscripts are significantly different ($P < 0.05$).
[1] SR (%): Survival rate (%) = 100 × (final number of fish) / (initial number of fish).
[2] WGR (%): weight gain rate (%) = 100 × (final total weight (g) − initial total weight (g)) / initial total weight (g).
[3] FCR: feed conversion ratio = dry diet fed (g)/wet weight gain (g).
[4] PER: protein efficiency ratio = wet weight gain/protein intake.
[5]HSI (%): hepatosomatic index (%) = 100 × liver weight (g) of final individual fish /final individual weight (g).

Body composition of fish is presented in Table 4. No significant differences were found in whole-body ash, protein, and lipid contents of fish fed the different experimental diets.

Table 4. Effects of different dietary levels of vitamin C on whole body composition and muscle and liver vitamin C concentrations of pufferfish

Dietary vitamin C levels (mg/kg)	*2.6*	*48.9*	*95.5*	*189.83*	*382.4*	*779.53*
Protein (%)	22.7±0.40	22.95±0.80	23.4±0.46	23.65±0.82	23.58±0.66	22.71±0.76
Lipid (%)	59.24±1.57	60.24±1.37	60.24±0.87	59.78±1.43	60.78±1.61	60.78±1.31
Ash (%)	10.02±0.51	9.38±0.18	10.12±0.73	9.91±0.37	9.92±0.37	9.93±0.45
vitamin C concentrations in	20.56±0.50^{a}	37.56±0.69^{b}	58.73±0.87^{c}	74.23±0.81^{d}	103.36±2.68^{e}	101.23±2.12^{e}
vitamin C concentrations in muscle(mg/kg)	6.33±0.26^{a}	9.03±0.44^{b}	20.56±0.58^{c}	22.33±0.54^{c}	32.93±1.30^{d}	32.3±0.88^{d}

Data are means ± SE (n = 3). Means in the same row with different superscripts are significantly different ($P<0.05$)

Vitamin C concentrations in liver and muscle are shown in Table 4. Vitamin C content in liver and muscle significantly increased ($P<0.05$) as dietary vitamin C increased. The broken-line regression analysis showed that the vitamin C requirement of pufferfish was 519.17 and 583.33 mg/kg diet based on liver (Fig. 1a) and muscle (Fig. 1b) vitamin C accumulation, respectively.

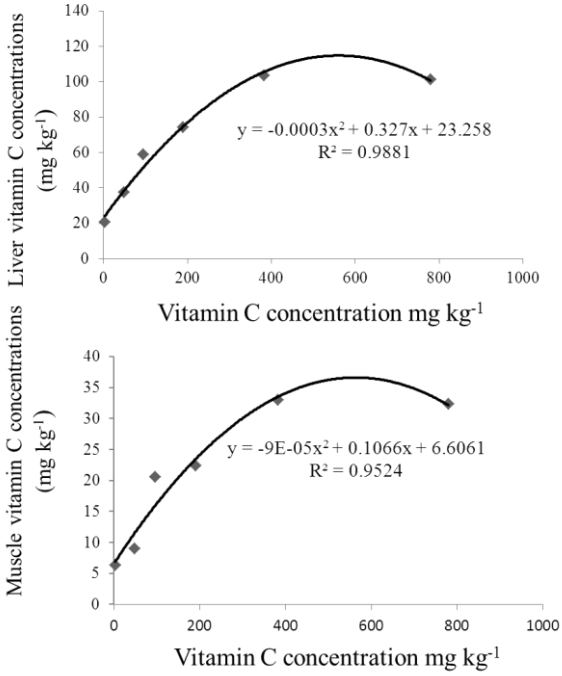

Fig.1. Polynomal regression analyses relationship of liver (a) and muscle (b) vitamin C concentrations to dietary vitamin C concentration.

After the feeding trial, the blood cell count in pufferfish fed dietary vitamin C were significant higher than in pufferfish fed the control diet (Fig. 2a). Significantly higher respiratory burst activity was found in fish fed diets with vitamin C (Fig. 2b).

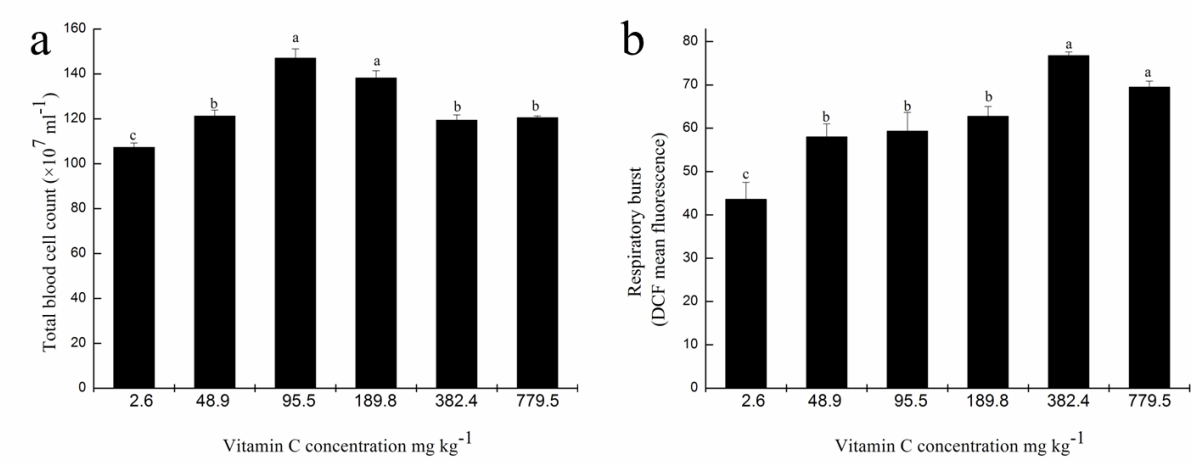

Fig.2. Effects of different dietary levels of vitamin C on total blood cell count (a) and respiratory burst (b) of pufferfish. Results are expressed as mean ± SD for triplicate samples. Asterisks indicate results that are significantly different from the control ($P < 0.05$).

Plasma alkaline phosphatase (AKP), cholesterol (CHOL), glutamic-pyruvic transaminase (GPT), and glutamic-oxaloacetic transaminase (GOT) of pufferfish are shown in Table 5. There were no significant variations in plasma CHOL of pufferfish among different dietary vitamin C concentrations ($P>0.05$). The plasma GLU, GPT, and GOT levels were significantly lower in fish fed vitamin C diet compared to fish fed the control diet ($P<0.05$). Compared with the control diet, the diets supplemented with vitamin C increased plasma AKP activity ($P<0.05$).

Table 5. Effects of different dietary levels of vitamin C on hematological parameters of pufferfish.

Dietary vitamin C levels(mg/kg)	*2.6*	*48.9*	*95.5*	*189.83*	*382.4*	*779.53*
AKP(U/mL)	88.89±2.48^{a}	111.55±2.62^{b}	140.50±3.64^{c}	151.48±9.03^{c}	145.53±5.32^{c}	142.92±2.93^{c}
GPT (U/mL)	25.21±0.56^{d}	20.17±0.55^{c}	15.49±0.45^{a}	14.85±0.84^{a}	19.80±0.19^{c}	17.46±0.56^{b}
GOT (U/mL	117.81±5.20^{b}	77.04±8.1^{a}	85.85±4.24^{a}	75.06±2.56^{a}	86.33±5.23^{a}	77.31±5.21^{a}
CHOL(mmol/mg)	3.21±0.20	2.50±0.25	2.90±0.25	2.62±0.35	2.76±0.24	3.32±0.31

Data are means ± SE (n = 3). Means in the same row with different superscripts are significantly different (P<0.05).

Malondialdehyde (MDA), alkaline phosphatase (AKP), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) activity in liver are shown in Table 6. MDA in fish fed diets supplemented with vitamin C was significantly lower than in fish fed the control diet (P < 0.05). SOD activity was significantly influenced by the dietary vitamin C levels. Higher activity of GPx was displayed in diets with increased vitamin C levels. CAT activity significantly increased with dietary vitamin C, and the highest value was found to be 12.74 ± 0.55 in the treatment with 189.83 mg/kg of vitamin C. However, no significant difference was observed in AKP activity among dietary treatments (P>0.05).

Table 6. Effects of different dietary levels of vitamin C on hepatic enzyme activity of pufferfish.

Dietary vitamin C levels(mg/kg)	*2.6*	*48.9*	*95.5*	*189.83*	*382.4*	*779.53*
MDA (nmol/mg)	0.182±0.004^{e}	0.160±0.003^{d}	0.131±0.003^{c}	0.089±0.005^{b}	0.073±0.004^{a}	0.067±0.004^{a}
SOD (U/mL)	20.64±0.50^{a}	24.66±0.09^{b}	25.78±0.53^{bc}	26.40±0.27^{c}	26.63±0.51^{c}	26.3±0.28^{c}
AKP(U/mL)	0.45±0.11	0.43±0.12	0.51±0.13	0.55±0.05	0.54±0.10	0.51±0.17
GPx (U/mL)	1.3±0.048^{a}	1.63±0.032^{b}	2.10±0.067^{cd}	2.25±0.060^{d}	1.98±0.077^{c}	2.05±0.078^{cd}
CAT (U/mL)	5.77±0.34^{a}	7.95±0.37^{b}	10.61±0.62^{c}	12.74±0.55^{d}	12.34±0.45^{cd}	10.94±0.34^{c}

Data are means ± SE (n = 3). Means in the same row with different superscripts are significantly different (P<0.05).

The relative expression levels of growth-related genes in liver of pufferfish fed with different levels of dietary vitamin C are presented in Fig. 3. Compared with the control diet, growth hormone receptors (GHR) and Insulin-like growth factor (IGF-1), gene expression levels were up-regulated in fish fed vitamin C diet (P < 0.05). Moreover, the highest levels of GHR and IGF-I were observed in fish fed 95.5 mg/kg vitamin C diet.

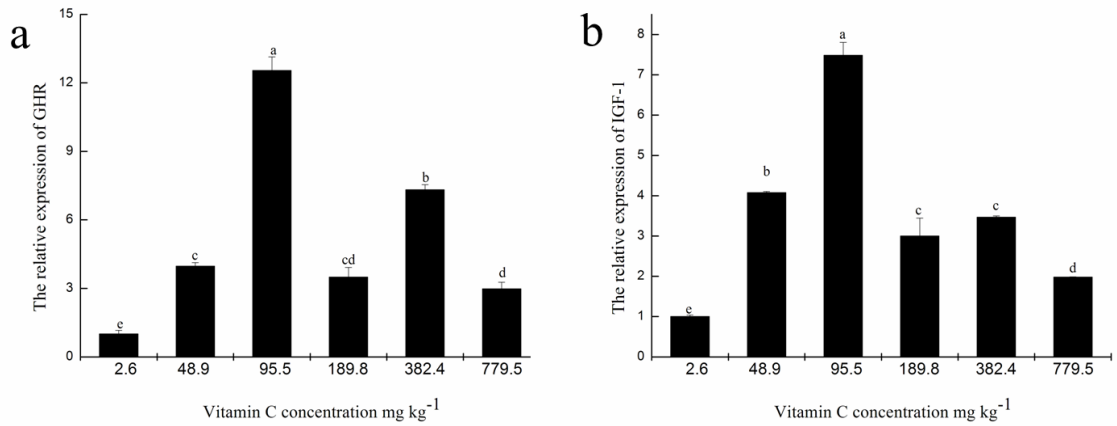

Fig.3. Effects of different dietary levels of vitamin C on relative expression levels of GHR (a) and IGF-1(b) of pufferfish. Results are expressed as mean ± SD for triplicate samples. Asterisks indicate results that are significantly different from the control (P < 0.05).

The effects of vitamin C on expression of immune-related genes (HSP70, HSP90, BAFF, TNF-α and IL-6) are shown in Fig. 4. The relative expression levels of HSP70 and HSP90 were up-regulated in fish fed vitamin C diet ($P<0.05$). The present study revealed that feeding on vitamin C dietary significantly increased the expression of BAFF, TNF-α, and IL-6 genes. The highest levels of BAFF, TNF-α, and IL-6 were observed in fish fed 95.5 mg/kg vitamin C diet.

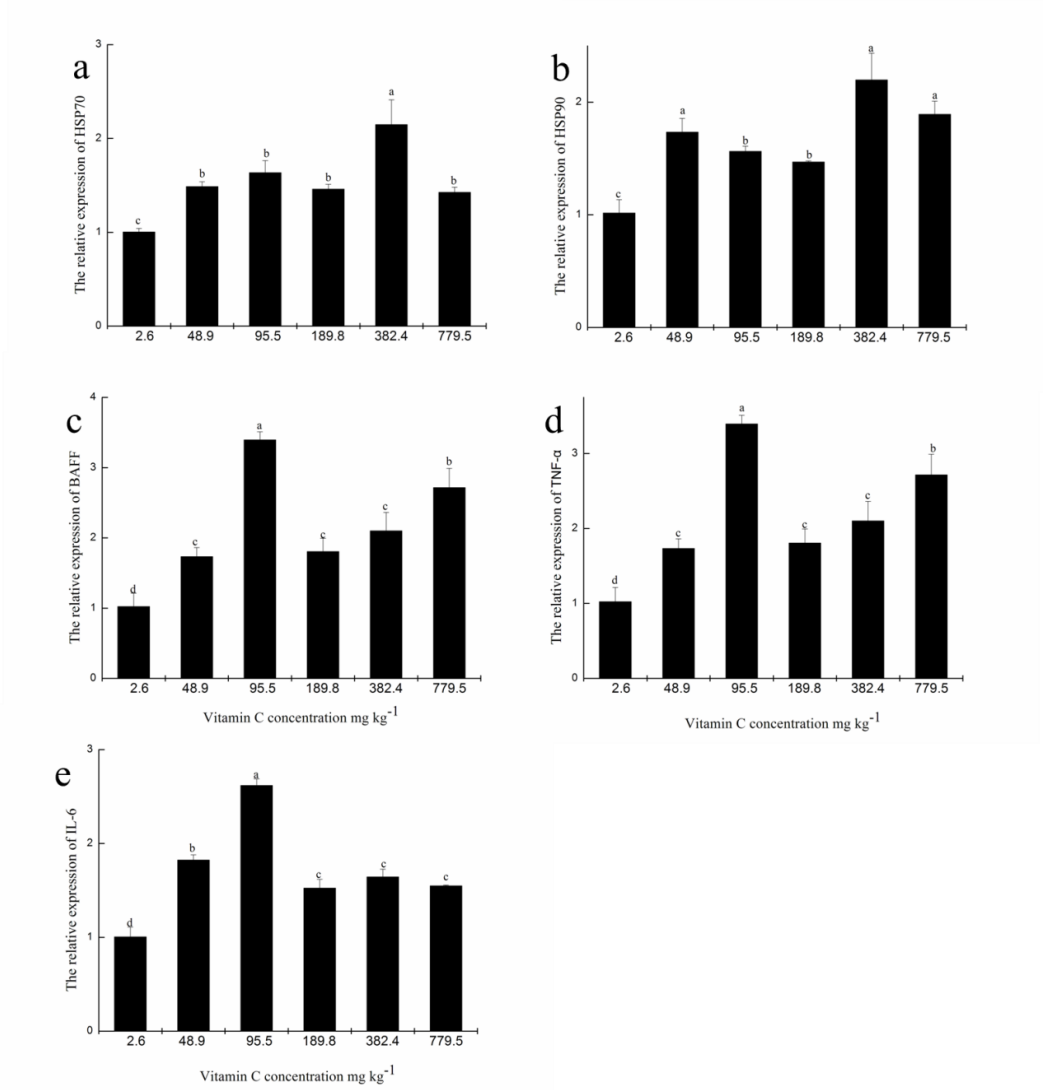

Fig.4. Effects of different dietary levels of vitamin C on relative expression levels of HSP70 (a), HSP90 (b), BAFF (C), TNF-α (d) and IL-6 (e) of pufferfish. Results are expressed as mean ± SD for triplicate samples. Asterisks indicate results that are significantly different from the control ($P < 0.05$).

Discussion

Vitamin C is an essential nutrient to maintain the normal physiological functions of fish. Dietary vitamin C can increase Weight gain rate (WGR) and protein efficiency ratio (PER), and decrease feed conversion ratio (FCR) of pufferfish. There were no significant differences in WGR, PER, and FCR among all the diets supplemented with vitamin C. These results indicate that dietary vitamin C improves growth performance and feed utilization of pufferfish. This is consistent with results from previous studies on other fish species (Fracalossi et al., 2001; Ai et al., 2006; Wang et al., 2003).

The growth hormone/insulin-like growth factors (GH/IGF) axis has been considered as an important neuroendocrine parameter regulating growth in fish (Berryman et al., 2008). Growth hormone receptors (GHR) and Insulin-like growth factor (IGF-1) are known to be key factors in the control of GH/IGF axis in teleosts. The physiological actions of growth hormone (GH) are triggered by binding to GHR on target tissues.

Association of GH with GHR activate a post-receptor signaling system that stimulates the transcription of target genes such as IGF-1 (Zhong et al., 2012). IGF-1 then can act on target tissues to stimulate cell proliferation, differentiation, and ultimately body growth (Pierce et al., 2005). The GH/IGF-1 axis is governed by the nutritional state of the fish (Pérez-Sánchez and Le-Bail, 1999). In the present study, GHR, and IGF-1 gene expression levels were up-regulated in fish fed vitamin C diet. There was a significant positive correlation between hepatic GHR and IGF-1 mRNA levels, and growth in fish (Vera-Cruz and Brown, 2009).

Hepatosomatic index (HSI) is used as an indicator of change in nutritional and energy status (Goede et al., 1990). In our study, HSI was lower in fish fed diets with 189.83 mg/kg and 382.4 mg/kg vitamin C than that in the other groups, suggesting that vitamin C decreased lipid accumulation in liver. This result is in agreement with a previous report (Chen et al., 2015). However, the HSI value of pufferfish was much higher than previous studies (2.1–3.1) on other fish species (Zhou et al., 2012; Wang et al., 2002); this might be attributed to its larger liver compared to other fish species.

Hepatic vitamin C concentration is usually considered the most sensitive in evaluating vitamin C status (Zhou et al., 2012). Liver vitamin C less than 20 mg/kg was an indicator of vitamin C deficiency in rainbow trout (Sato et al. 1982). In the present experiment, vitamin C concentrations in liver and muscle were significantly affected by the dietary vitamin C levels. Results are well documented in many other fish species (Zhou et al., 2012; Chen et al., 2015). Vitamin C concentrations in the liver (20.56-101.23 mg/kg) were much higher than those in the muscle (6.33-32.3 mg/kg). In another study vitamin C concentrations in the liver were higher than in the kidneys and muscle (Shahkar et al. 2015). The liver is an important organ in catabolism of vitamin C, and hepatic vitamin C saturation is a useful response criterion for determining its requirement. Generally, lower dietary requirement of vitamin C is needed to maintain normal physiological functions, and tissue storage is higher than that needed to maintain maximal growth (Xiao et al., 2010).

The innate system of fish mainly consists of phagocytosis, encapsulation, nodule formation, antimicrobial peptides formation, and cell agglutination. Several external and internal factors can influence the activity of innate immune parameters (Magnadóttir, 2006). In the present study, respiratory burst activity was significantly improved by moderate dietary vitamin C supplementation, indicating that fish immune function was enhanced by dietary vitamin C. Respiratory bursts produced by phagocytes have been widely used to evaluate the defense ability against pathogens. However, the excessive accumulation of respiratory burst activity can damage cell structure and function. Respiratory burst activity of yellow croaker was affected by dietary vitamin C levels (Ai et al. (2006).

In the present study, fish fed diets supplemented with 48.9-779.53 mg/kg vitamin C had higher AKP activity compared to fish fed the control diet. AKP has been reported to be involved in immune defense mechanisms, and is correlated with immune competence. AKP is an important enzyme that regulates some essential functions in all living organisms. High AKP activity in scallops indicated a strong immuno-competent ability (Xing et al. 2002). Vitamin C has also been shown to enhance AKP activity in the Serum of juvenile cobia. These results suggest that vitamin C can stimulate innate immune responses in pufferfish. In our study, plasma GOT, and GPT activity decreased with the supplementation of vitamin C, indicating improved results in the liver when dietary vitamin C levels increased. When the liver is damaged, GOT and GPT will be released into the blood, elevating blood transaminase activity. Therefore, plasma GOT and GPT are used as indicators of liver function. This supported the results of a study which found that feeding higher dietary vitamin C supplementation reduced GOT level in Japanese flounder (Gao et al. 2014). This may be associated with the antioxidant function of vitamin C which stabilized biological membranes and protected cells against damage (Liu et al., 1995).

Malondialdehyde (MDA) is the main component of lipid peroxidation, but excessive MDA can damage cell structure and function. In the present study, hepatic MDA in fish fed

diets supplemented with vitamin C significantly decreased, compared to fish fed the control diet. The results suggest that increased vitamin C can reduce the level of peroxidation in tissues. In our study, the treatments supplemented with vitamin C increased hepatic antioxidant enzyme (SOD, GPx, CAT) activity. SOD can catalyze the reaction of super anion transforming it to H_2O_2 and O_2. GPx and CAT are ROS scavenger enzymes, which can decompose H_2O_2 into O_2 and H_2O. Therefore, the antioxidant status in fish can accurately reflect the activity of SOD, GPx, and CAT. Wuchang bream fed vitamin C had significantly enhanced hepatic SOD activity (Ming et al. 2012). These results indicated that vitamin C can enhance the antioxidant system in pufferfish.

Heat shock proteins (HSPs), known as stress proteins and extrinsic chaperones, play a critical role in numerous cellular functions and immune response. The HSP family consists of HSP60, HSP70, HSP90, HSP100, and other low-molecular mass HSPs. In the present study, levels of hepatic HSP70 and HSP90 mRNA increased with dietary vitamin C, indicating that dietary vitamin C can enhance the expression of heat shock proteins. Enhanced levels of HSP70 and HSP90 in fish may reflect a protective response against environmental pollutant-related stress. Previous studies found that vitamin C can increase the expression levels of HSP70 in Wuchang bream (Ming et al., 2012). It was found that a higher level of dietary vitamin C (829.8 mg/kg) can increase the mRNA levels of HSP70 and HSP90 (Wu et al. 2014). However, another report showed that vitamin C can decrease the expression of HSP70 in broilers (Mahmoud, 2004).

In the present study, we found that vitamin C can increase cytokines (BAFF, TNF-α and IL-6) expression, indicating that vitamin C might improve immunomodulatory function in pufferfish. BAFF, TNF-α, and IL-6, are important cytokines which can regulate innate immunity. BAFF is considered an important member of the TNF family of cytokines and plays a major role in B-cell survival, proliferation, and differentiation (Ai et al., 2011). It is mainly produced by innate immune cells such as macrophages/monocytes, neutrophils, dendritic cells, activated T-cells, and malignant B-cells (Mackay et al., 2002). TNF-α is a crucial regulator and effector in the process of mounting innate and adaptive immune responses against pathogens, by regulating cell death and survival (Locksley et al., 2001; Niu et al., 2014). IL-6 is a pleiotropic inflammatory cytokine involved in numerous biological functions including oncogenesis, inflammation, immune regulation, and hematopoiesis (Wang and Secombes, 2009). Expression levels of IL-1 band TNF-α were influenced by external factors including nutrition in fish (Kiron, 2012). Vitamin E-supplemented diet was found to increase expression levels of IL-1β and TNF-α (Niu et al. (2014). It was found that dietary laminarin supplementation significantly increased the mRNA levels of IL-1β, IL-8 and TLR2 (Yin et al. 2014).

In conclusion, the present study indicated that moderate doses of dietary vitamin C can significantly enhance growth, non-specific immunity, and antioxidant capability of pufferfish. Moreover, the present study provided the first information concerning vitamin C induced growth-related genes (GHR and IGF-1) and immune-related genes (BAFF, TNF-α and IL-6) expression in fish. These findings are helpful to understand the effects of dietary vitamin C on growth performance, antioxidant status, and innate immunity, in pufferfish.

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