



Effects of Dietary Inclusion of Vitamin C on Immune and Antioxidant Defence System in Captive Reared Juvenile Mahseer, *Tor putitora* (Hamilton, 1822)

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Abstract

The present trial was conducted to evaluate the effects of dietary L-ascorbyl-2-polyphosphate (vitamin C) on immune and antioxidant defence system in captive-reared juvenile mahseer (*Tor putitora* (Hamilton, 1822)). The experiment consisted of four treatments (3 replicates each), which received four semi-purified diets containing 0, 100, 200 and 300 mg.kg⁻¹ of vitamin C. A total of 120 fish with an average initial body weight, 2.27 ± 0.01 g were distributed among 12 tanks (10 fish per tank). At the end of the 70-day experiment, all fish of each group were subjected to hypoxia stress for 48 h. Fish fed 300 mg.kg⁻¹ vitamin C showed significantly increased ($P < 0.05$) serum lysozyme activity in response to hypoxia stress, followed by 200 and 100 mg.kg⁻¹ vitamin C. Similar results were observed for glutathione peroxidase (GSH-Px), glutathione reductase (GSR) and superoxide dismutase (SOD) activity. After hypoxia, malondialdehyde (MDA) concentration was considerably reduced ($P < 0.05$) in fish fed 300 mg.kg⁻¹ vitamin C. These results indicated that in stressful conditions feeding diets supplemented with 300 mg.kg⁻¹ vitamin C would be better than 200 and 100 mg.kg⁻¹ vitamin C, to obtain the optimum immune and antioxidant status of captive-reared juvenile mahseer.

Keywords: L-ascorbyl-2-polyphosphate, immune-biochemical aspects, fish well-being, fish nutritional physiology

Introduction

Adequate amount of dietary vitamin C is essential for proper physiological and biochemical functions of the body (Dabrowski 1990, 2001; Zhou et al. 2012; Chen et al. 2015). During stressful conditions, its supplementation helps in the elimination of stress and improves the immunity and antioxidant status of fish (Eo and Lee 2008; Tewary and Patra 2008). However, vitamin C scarcities cause a decline in the well-being of fish (Gouillou-Coustans and Kaushik 2000).

The innate immunity and antioxidant defence system play an important role against stress in all animals including fish (Saurabh and Sahoo 2008; Zhou et al. 2012). During normal conditions a balance exists between the production and elimination of reactive oxygen species (ROS) such as hydrogen peroxides, superoxide anion radicals, and hydroxyl radicals, however, during stressful conditions, the balance disturbs and causes oxidative stress which exerts negative effects on various components of the body

cells, cellular membranes and inner components of the cells including lipids, nucleic acids, and proteins (Valavanidis et al. 2006). Consequently, immune and defence systems stimulate and provide necessary protection to the body against stress (Oost et al. 2003; Ai et al. 2006; Ullah 2012).

Unfavourable aquatic environments are the main causes of oxidative stress (Balm 1997; Ullah 2012). Being a strong antioxidant, vitamin C has the capability of eliminating oxidative stress (Fetoui et al. 2008). Supplementation of vitamin C is getting popular in fish diets (Gabaudan and Verlhac 2001; Kiron et al. 2004; Korkmaz et al. 2009). Mahseer is an important freshwater fish species in the aquaculture industry in Trans-Himalayan countries. It is currently facing a threatening situation in its natural territory due to increasing aquatic pollution, habitat destruction and overfishing (Islam and Tanaka 2004). For successful and healthy production of mahseer in captive environments, the formulation of diets with optimum nutritional requirements is crucial. Supplementation of high levels of dietary vitamin C besides exerting

beneficial effects on growth and feeding parameters of mahseer (Khan et al. 2015), also showed significant positive effects on physio-biochemical aspects of the species under study. The present study aimed to evaluate the effects of different levels of dietary vitamin C on immune and antioxidant defence system in captive-reared *T. putitora* juveniles in stressful conditions.

Materials and Methods

Acclimatisation and experimental diets

Tor putitora juveniles were collected from the Attock fish hatchery and transferred to the Laboratory of Fisheries and Aquaculture at the Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan. All fish were stocked in two large size fibre tanks with water capacity of 1,000 L and subjected to one-week acclimatization while offering them a vitamin C free basal diet. The formulation and composition of basal diet is shown in Table 1. The required feed ingredients were obtained from the National Agricultural Research Centre (NARC), Islamabad. Four semi-purified diets were prepared i.e., a control (vitamin C free) while other three diets contained vitamin C at the rate of 100, 200 and 300 mg.kg⁻¹. L-ascorbyl-2-polyphosphate (Langen Suzhou, China) was used as a source of dietary vitamin C. The chemicals used in analyses of the immune-biochemical aspects performed during the present study were purchased from Sigma-Aldrich, United States.

The required ingredients were weighed and mixed well, then ground, and appropriate amounts of oil and water were added. The dough mixtures for all four diets were extruded through a meat grinder and the resulting pellets were dried at 55 °C for 24 h, then stored at -20 °C until use.

Table 1. Body formulation and composition of basal diet.

Ingredients	Amount g.kg ⁻¹
Soybean meal	130
Wheat bran	10
Fish meal	250
Wheat flour	10
Gluten 60 %	500
Sunflower meal	50
Rice bran	10
*Premix	10
Di-calcium phosphate (DCP)	10
Canola oil	20
Total	1,000 g

Basal diet was composed of 40 % crude protein, 6 % total fat and 4.5 % crude fibre.

*Premix was Vitamin C free and consisted of vitamins, minerals and essential amino acids including Vitamin A

(retinol, retinal, retinoic acid and beta-carotene) 40,000,000 IU; Vitamin D₃ (cholecalciferol) 820,000 IU; Vitamin E (tocopherol) 6200 mg; Vitamin K₃ (menadione) 800 mg; Vitamin B₂ (riboflavin) 2500 mg; Vitamin B₃ (niacin) 5,100 mg; Vitamin B₁₂ (cobalamin) 1000 mg; Choline chloride 125,500 mg; Manganese 30,000; Zinc 17,555 mg; Copper 1,000 mg; Cobalt 50 mg and Iodine 300 mg and L-lysine 10,500 mg; DL-methionine 50,500 mg.

Composition of diets

The basal diet consisted of 40 % crude protein and 6 % total fat and ascorbic acid concentrations (AA) analysed to be 1.5, 98.1, 196.5 and 295.9 mg.kg⁻¹ in the four test diets such as vitamin C0, C100, C200 and C300, respectively.

Experimental design, fish and water quality

The experimental design was completely randomized, consisted of four treatments with three replicates each. A flow-through system was used which connected with 12 fibre tanks (each with water capacity of 90 L) provided aerated freshwater. After one-week adaptation, 10 uniform size mahseer fish (initial average live body weight, 2.27 ± 0.01 g) were stocked per tank/replicate. The fish were fed the respective experimental diets daily at 6 % body weight at 08:00, 12:00 and 17:00 h.

The experimental tanks were cleaned once a week to remove the faeces and uneaten feed. During the trial, water quality parameters such as, average pH (7.4 ± 0.2), average temperature (27.0 ± 0.4 °C) and average dissolved oxygen (6.0 ± 0.2 mg L⁻¹) were measured each day and found at normal levels for the species under study. Moreover, water samples were collected once a week for the measurement of total ammonia (average ammonia, 0.19 ± 0.3 mg L⁻¹).

Hypoxia stress test

At the end of the 70-day trial, fish that received different levels of dietary vitamin C were subjected to hypoxia (50 % saturation) stress test. The oxygen supply was reduced up to 50 % saturation in all the experimental tanks for a period of 48 h to check the immune and antioxidant responses.

Sample collection

After hypoxia stress test, fish of all groups were immediately anaesthetized with MS222 (tricaine methanesulfonate) solution (60 mg.L⁻¹). A total of 2 mL blood was taken through tail ablation method and centrifuged at 10,000 revolutions per min (rpm) for 15 min (Khan et al. 2016, 2017). Serum was separated and stored at -20 °C for the analysis of lysozyme activity. Then the fish were immediately placed in ice box and

the fish were then dissected to remove 5 gm of muscle and 5 gm of liver from each fish. Tissue samples were quickly frozen in liquid nitrogen and then stored in a freezer at -20°C for the analysis of immuno-biochemical aspects.

Immune-biochemical analyses

Serum lysozyme enzyme activity ($\mu\text{g}\cdot\text{mL}^{-1}$) was measured according to the guidelines of Anderson and Siwicki (1994). A 100 μL sample of serum was used to measure the lysozyme activity. Glutathione peroxidase (GSH-Px) enzyme activity was determined according to the method of Mohandas et al. (1984). Glutathione reductase (GR) enzyme activity was measured according to the descriptions of Carlberg and Mannervik (1975). The guidelines of Kakkar et al. (1984) were followed for the measurement of superoxide dismutase (SOD) enzyme activity. Malondialdehyde (MDA) concentration was measured according to the method of Wright et al. (1981) and Iqbal et al. (1996). Ascorbic acid (AA) concentrations in test diets and in liver of fish were estimated according to the "2, 4-dinitrophenylhydrazine" colourimetric method as described by Dabrowski and Hinterleitner (1989). Liver protein concentrations were determined according to Lowry's method (1951).

Statistical analysis

The programme "Statistix 8.1 analytical software" was used for the analysis of data. The data were submitted to the analysis of variance (One-way ANOVA). When a significant variance was observed, Tukey LSD (least significant difference) test was applied to compare the averages. The difference was measured as significant at ($P < 0.05$).

Results

Immune and antioxidant defence system

Vitamin C supplementation showed dose-dependent effects on the immune and antioxidant defence system of mahseer. After hypoxia stress test, fish fed the basal diet, presented significantly lower ($P < 0.05$) immune-biochemical parameters than those fed vitamin C supplemented diets. Fish fed diet supplemented with high-level vitamin C ($300\text{ mg}\cdot\text{kg}^{-1}$) showed significantly increased ($P < 0.05$) serum lysozyme activity, followed by those fed 200 and 100 $\text{mg}\cdot\text{kg}^{-1}$ vitamin C (Fig. 1).

Fish fed $300\text{ mg}\cdot\text{kg}^{-1}$ vitamin C for 70 days when subjected to hypoxia-induced stress, showed significantly increased ($P < 0.05$) GSH-Px and GR (Table 2) and SOD (Table 3) enzyme activities, followed by those fed 200 and 100 $\text{mg}\cdot\text{kg}^{-1}$ vitamin C.

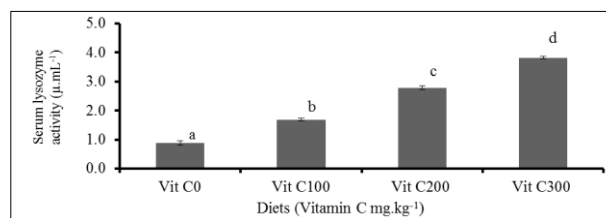


Fig. 1. Sampling Effect of different levels of dietary vitamin C on serum lysozyme activity in *Tor putitora* juveniles.

Table 2. Body length and gonad parameters of female ayu in the Tien Yen River.

Diets	GSH-Px (liver)	GR (liver)	GSH-Px (muscle)	GR (muscle)
Vit C ₀	1.18 ± 0.08 ^d	823.01 ± 3.05 ^d	1.08 ± 0.02 ^d	813.01 ± 2.11 ^d
Vit C ₁₀₀	1.42 ± 0.04 ^c	860.17 ± 1.09 ^c	1.45 ± 0.06 ^c	851.24 ± 3.05 ^c
Vit C ₂₀₀	1.58 ± 0.06 ^b	874.06 ± 1.79 ^b	1.57 ± 0.01 ^b	864.33 ± 1.14 ^b
Vit C ₃₀₀	2.23 ± 0.04 ^a	911.68 ± 2.04 ^a	2.04 ± 0.03 ^a	909.49 ± 3.05 ^a

Values are represented as average ± standard error (n = 3). Averages represented by different letters within the same column are significantly different ($P < 0.05$).

Table 3. Effect of different levels of dietary vitamin C on superoxide dismutase enzyme activity ($\mu\text{moles min}^{-1}\text{ mg}^{-1}$ protein) in *Tor putitora* juveniles.

Diets	Liver tissue	Muscle tissue
Vit C ₀	631.23 ± 5.13 ^d	483.13 ± 11.23 ^d
Vit C ₁₀₀	678.27 ± 9.77 ^c	503.10 ± 8.83 ^c
Vit C ₂₀₀	691.27 ± 8.05 ^b	521.80 ± 8.50 ^b
Vit C ₃₀₀	750.78 ± 3.74 ^a	611.19 ± 5.94 ^a

Values are represented as average ± standard error (n = 3). Averages represented by different letters within the same column are significantly different ($P < 0.05$).

A significant elevation ($P < 0.05$) was observed in the MDA concentrations in liver and muscle tissues of fish fed basal diet after subjected to stress as compared to those fed diets supplemented with vitamin C at the rates of 300, 200 and 100 $\text{mg}\cdot\text{kg}^{-1}$ (Table 4).

Table 4. Effect of different levels of dietary vitamin C on malondialdehyde ($\mu\text{mole min}^{-1}\text{ mg}^{-1}$ protein) concentrations in *Tor putitora* juveniles.

Diets	Liver tissue	Muscle tissue
Vit C ₀	2.09 ± 0.03 ^d	2.25 ± 0.08 ^d
Vit C ₁₀₀	1.63 ± 0.01 ^c	1.82 ± 0.03 ^c
Vit C ₂₀₀	1.43 ± 0.02 ^b	1.60 ± 0.06 ^b
Vit C ₃₀₀	0.71 ± 0.01 ^a	0.97 ± 0.05 ^a

Values are represented as average ± standard error (n = 3). Averages represented by different letters within a column are significantly different ($P < 0.05$).

Liver ascorbic acid (AA) and protein concentration

Fish fed vitamin C at the rate of 300 mg.kg⁻¹ diet showed a significantly improved ($P < 0.05$) liver AA and protein concentrations after hypoxia stress, followed by 200 and 100 mg.kg⁻¹ vitamin C, than fish fed basal diet (Table 5).

Table 5. Effect of different levels of dietary vitamin C on ascorbic acid and total protein concentration in liver tissue of *Tor putitora* juveniles.

Diets	Liver AA content ($\mu\text{g.g}^{-1}$)	Liver total protein content (mg.g^{-1})
Vit C ₀	08.93 \pm 0.01 ^d	06.47 \pm 0.35 ^d
Vit C ₁₀₀	26.68 \pm 0.02 ^c	22.58 \pm 0.55 ^c
Vit C ₂₀₀	38.92 \pm 0.05 ^b	32.1 \pm 0.11 ^b
Vit C ₃₀₀	69.34 \pm 0.08 ^a	57.54 \pm 0.35 ^a

Values are represented as average \pm standard error ($n = 3$). Averages represented by different letters within the same column are significantly different ($P < 0.05$).

Correlation between dietary vitamin C and liver ascorbic acid concentrations

A positive correlation was found between liver AA concentrations and dietary vitamin C levels in mahseer fish (Fig. 2) such that the liver AA concentrations positively correlated the dietary levels of vitamin C.

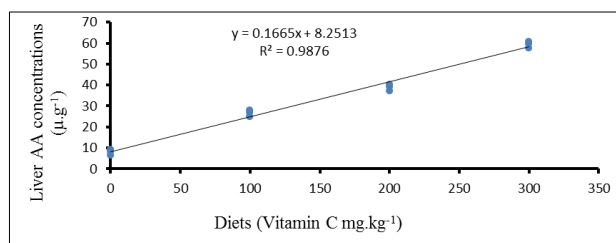


Fig. 2. Positive correlation between different levels of dietary vitamin C and liver ascorbic acid concentrations in *Tor putitora* juveniles.

Discussion

The present significant immune-biochemical responses of mahseer fish to high level of dietary vitamin C are strongly in accordance to our previous results obtained for the growth and feeding aspects of the same fish in response to dietary vitamin C (Khan et al. 2015).

Lysozyme (N-acetylmuramidase or muramidases), a hydrolytic enzyme is considered an important component of the immune system and is found abundantly in all animals including fish (Misra et al. 2007; Saurabh and Sahoo 2008). It is released from the leukocyte cells and plays a significant role in the

antimicrobial activity in animal body. Several authors have reported that ascorbic acid supplementation significantly improves the immune system by increasing the lysozyme and bactericidal activity (Ai et al. 2006; Misra et al. 2007; Zhou et al. 2012). In gilt-head sea bream (*Sparus aurata* Linnaeus, 1758), the immune response was boosted in terms of increased bactericidal activities after 8 weeks of dietary ascorbic acid supplementation (Ortuno et al. 1999). Eo and Lee (2008) observed that tiger puffer (*Takifugu rubripes* Temminck & Schlegel, 1850) fed vitamin C supplemented diet had considerably elevated lysozyme activity than fish fed vitamin C free diet.

Lysozyme activity has been shown to be modified by several environmental factors such as, low water quality, pollution, toxicants, poor management and handling of fish, and oxidative stress. Thus, it is useful to mix vitamin C supplementation in fish feeds for maintaining their proper immunity status by improving the lysozyme activity (Goede and Barton 1990). Ibrahem et al. (2010) observed that supplementation of elevated level of vitamin C (500 mg.kg⁻¹) after one and couple of months considerably increased the lysozyme activity in Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758). Similar results were observed in our present study where the supplementation of high-level vitamin C (300 mg.kg⁻¹) considerably increased ($P < 0.05$) the serum lysozyme activity in *T. putitora*, followed by 200 and 100 mg.kg⁻¹ vitamin C compared to the basal diet.

During stress, the production of malondialdehyde (MDA) reaches the toxicity levels and starts damaging body cells (Freeman and Crapo 1982; Sies 1997). Meanwhile, the body defence system activates and produces several important enzymes such as superoxide dismutase, glutathione peroxidase, and glutathione reductase which play an important role in the degradation of reactive oxygen species and thus reduce the oxidative stress (Tabrez and Ahmad 2009). In the present study, the activities of SOD, GSH-Px, GR and MDA concentrations were examined in *T. putitora* juveniles exposed to hypoxia stress after they were fed different levels of dietary vitamin C. The vitamin C supplementation showed positive effects on the antioxidant status by increasing the production of antioxidant enzymes while reducing the MDA concentrations. These results are in agreement with those of Chen et al. (2015), Hu et al. (2013) and Asaikkutti et al. (2016). The juvenile yellow catfish (*Pelteobagrus fulvidraco* (Richardson, 1846)) fed high-level vitamin C showed higher antioxidant enzyme (CAT, SOD and GSH-Px) activities as compared to those fed vitamin C free diet (Liang et al. 2017). Similarly, juvenile cobia (*Rachycentron canadum* (Linnaeus, 1766)) fed vitamin C supplemented diets had higher SOD activity as compared to basal diet (Zhou et al. 2012). Ullah (2012) noted that high-level vitamin C supplementation (200 mg.kg⁻¹) has a strong protective role against oxidative stress caused by the hyperoxic and hypoxic conditions in juvenile silver

carp (*Hypophthalmichthys molitrix* (Valenciennes, 1844)).

Malondialdehyde (MDA) is produced as a result of lipid peroxidation and is considered as an important oxidative stress marker (NRC 2011). Vitamin C supplementation in diets has protective effects in all animals including fish against stress (Krishnamoorthy and Sangeetha 2008; Ullah 2012). Yellow catfish fed vitamin C free diet had greater MDA levels as compared to vitamin C supplemented diets (Liang et al. 2017). Vitamin C supplementation *in vivo* may help in repairing the vitamin E (Chan 1993) which consequently enhance the antioxidant activities while reduces the MDA concentrations. Similar results have been observed in thornfish (*Terapon jarbua* (Forsskål, 1775)) (Chien and Hwang 2001) and juvenile cobia (*R. canadum*) (Zhou et al. 2012). These results support the present work which concludes that supplementation of high level dietary vitamin C (300 mg.kg⁻¹) considerably reduces the MDA concentration in *T. putitora* juveniles, followed by 200 and 100 mg.kg⁻¹ vitamin C, than basal diet.

Total protein content is directly related to animal health (Tewary and Patra 2008). In the present findings, total protein content (mg.g⁻¹) in liver tissue of *T. putitora* considerably increased ($P < 0.05$) when fed diet supplemented with high-level vitamin C (300 mg.kg⁻¹), followed by 200 and 100 mg.kg⁻¹ vitamin C than basal diet. Nsonga (2009) observed higher protein efficiency ratio and protein conversion efficiency in the juvenile tilapia (*Oreochromis karongae* (Trewavas, 1941)) fed diets supplemented with ascorbic acid as compared to control diet. Similar results were observed by Fracalossi et al. (1998) in juvenile Oscars (*Astronotus ocellatus* (Agassiz, 1831)) cichlids.

A positive correlation was observed in the present study between different levels of dietary vitamin C and liver AA concentrations (Fig. 2). A similar correlation has been determined between liver vitamin C concentrations and different levels of dietary ascorbic acid in rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792)) (Dabrowski 2001) and silver carp (Ullah 2012). Literature shows that decrease in liver AA concentrations has a negative impact on fish health. Atlantic salmon (*Salmo salar* Linnaeus, 1758) when cultured under abnormal oxygen supply for 12 weeks showed reduced tissue ascorbic acid concentrations (Lygren et al. 2000).

Conclusion

Low oxygen levels negatively affect the immune and antioxidant status and liver AA concentrations in fish. These results indicate that the supplementation of high level of dietary vitamin C eliminates the hypoxia-induced harmful effects of mahseer fish reared in intensive culture systems, improves its health and

helps in successful culture practices. Thus, according to the present results during the stressful conditions, feeding diets supplemented with 300 mg.kg⁻¹ vitamin C showed better results than 200 and 100 mg.kg⁻¹ vitamin C, for the successful production and obtaining optimum immune and antioxidant status of juvenile mahseer in captive environments.

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