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The ameliorative role of ascorbic acid against blood disorder, immunosuppression, and oxidative damage of oxytetracycline in rainbow trout (*Oncorhynchus mykiss*)

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Abstract This experiment was aimed to determine the possible beneficial effects of dietary ascorbic acid (AA) on hematological indices, immune responses, and antioxidative capacity of *Oncorhynchus mykiss* treated with antibiotic oxytetracycline (OTC). A total of 150 fish were divided evenly among five experimental groups (30 fish of each, in 3 replicates) receiving diets containing OTC (0 and 100 mg per kg fish weight) and AA (100, 200, 400, and 800 mg per kg fish diet) for 28 days. Treatments include group A or control (100 mg AA without OTC), group B (100 mg AA with OTC), group C (200 mg AA with OTC), group D (400 mg AA with OTC), and group E (800 mg AA with OTC). The results obtained showed that the hematological indices (red blood cells, white blood cells, hematocrit, hemoglobin, and neutrophils), immunological parameters (plasma lysozyme, plasma complement, and skin mucus alkaline phosphatase activities), and antioxidant enzymes activities (superoxide dismutase and catalase) were significantly decreased by OTC in *O. mykiss* fed control diet ($P < 0.05$). The results also revealed that OTC significantly increased the activity of biochemical enzymes (aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase) in the plasma of *O. mykiss* fed control diet ($P < 0.05$). However, in comparison to the control diet, feeding fish with higher amounts of AA (400 and 800 mg/kg diet) significantly restored the hematological, immunological, and antioxidative responses in OTC-treated groups ($p < 0.05$). These findings show that the dietary supplementation of AA at 400 or 800 mg/kg diet is beneficial in relieving *O. mykiss* from OTC-induced oxidative stress and immunosuppression.

Keywords Chemotherapy · Hematology · Oxidative status · Tetracycline · Vitamin C

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Introduction

During the previous decade, the outbreak of infectious diseases has emerged as a major limiting factor for aquaculture expansion which causes massive mortality and economic losses (Shalini et al. 2019). An important strategy used to address this problem is the administration of antibiotics (Rico et al. 2013). Tetracyclines are a common class of antibiotics which are widely used in aquaculture. Oxytetracycline (OTC) is a well-known tetracycline antibiotic

that is produced from *Streptomyces rimosus* fungi (Rodrigues et al. 2017a; FAO 1990). OTC is commonly used as an effective treatment against bacterial pathogens of fishes, such as *Aeromonas salmonicida*, *Aeromonas hydrophila*, *Lactococcus garvieae*, *Vibrio anguillarum*, and *Pseudomonas* sp. (Leal et al. 2019; Nakano et al. 2018). More utilization of OTC when compared with other antibiotics is due to its low-cost, legal availability, high efficiency, and non-specific selectivity in the treatment of bacterial infection (Pês et al. 2018; Lee et al. 2020; Rodrigues et al. 2017a). OTC can be applied via injection, immersion and oral administration, but is commonly administered through diet in the amount of approximately 75 mg per kg of fish biomass (Yonar 2012).

However, the administration of antibiotic drugs may pose several side effects such as the occurrence of antibiotic resistance in bacterial pathogens (Lunden et al. 1998), residue in fish tissues (Harikrishnan et al. 2009), and reduction of fish health (Shalini et al. 2019). For instance, OTC is found to induce oxidative stress and liver malfunctioning. OTC can increase the generation of reactive oxygen species (ROS) in the fish body, which cause peroxidation of liver cell membrane lipids resulting in an increased leakage of intracellular enzymes to the blood (Nakano et al. 2018). In addition, negative effects of OTC on immune response, antioxidant defense, hematological parameters, nephrotoxicity, genotoxicity, and histological changes in fish species have been previously documented (Rodrigues et al. 2017b; Hoseini and Yousefi 2019; Reda et al. 2013; Yonar et al. 2011; Mathew and Ambili 2017). As a result, OTC may increase the fish susceptibility to secondary pathogens and also to environmental stressors. Hence, there is a necessity for the discovery of methods to alleviate the harmful effects of OTC on fishes.

Vitamin C or L-ascorbic acid (AA) is an important micro-nutrient for fishes, playing a key role in the growth performance and physiological process (Lin and Shiau 2005). It is also a potent natural antioxidant that plays a vital role in scavenging ROS to protect the fish from oxidative stress (Bae et al. 2012; Hajirezaee et al. 2020). Moreover, AA has an important role in hematology (Sandnes et al. 1990), collagen formation (Hunter et al. 1979), reproduction (Emata et al. 2000; Cavalli et al. 2003), immune response (Misra et al. 2007; Roosta et al. 2014; Zhou et al. 2012), and recovery from exposure to toxicant and environmental stressors (Saha and Kaviraj 2009; Kim et al. 2017; Wahli et al. 2003). Dietary supplementation of AA can provide normal growth of fish at low doses; however, a higher dose of AA is required to increase the stress tolerance in fish (Vani et al. 2011).

Nevertheless, up to date, there is no data available on the role of dietary AA on OTC-induced stress in fish. Therefore, the current study was designed to assess the possible role of dietary AA in mitigating the adverse effects of OTC in terms of hematological parameters, skin mucus and blood immune parameters, and antioxidant capacity of rainbow trout (*Oncorhynchus mykiss*).

Materials and methods

Chemicals

Vitamin C (L-ascorbyl-2-monophosphate) and oxytetracycline were purchased from Sigma-Aldrich Co. (Darmstadt, Germany) and Rooyandarou Co. (Tehran, Iran), respectively. All the other chemicals were provided by Merck Chemical Co. (Darmstadt, Germany).

Experimental fish

Healthy juveniles of *Oncorhynchus mykiss* (43 ± 2.6 g) were purchased from a local fish farm and were transported to the wet lab in a fiberglass-aerated tank. They were acclimated to lab conditions for 14 days in 1000-L fiberglass tanks filled with tap water under a natural 12-h photoperiod. During the adaptation period, rainbow trout were fed three times a day with a commercial diet containing 43% crude protein, 14% crude fat, 4% crude fiber, and 11% ash. The water physicochemical parameters were regularly monitored, and water temperature range from 13.64 to 15.47 °C, pH from 7.2 to 7.5, and oxygen levels from 7.1 to 7.6 mg L^{-1}. Excess feed and fecal waste were siphoned out daily and a quarter portion of water in the tank is replaced with clean water.

Experimental diets

Five experimental diets were provided by mixing a basal diet (Table 1) with varying amounts of OTC (0 and 100 mg/kg body weight) and AA (100, 200,

Table 1 Composition and proximate analysis of the basal diet

Ingredients	Proportion (%)
Fish meal	50
Wheat flour	19.5
Soybean powder	12.73
Fish oil	6
Soybean oil	5
Dicalcium phosphate	0.5
Lecithin	0.5
Mineral premix[a]	3
Vitamin premix[b] (without ascorbic acid)	2
Butyl hydroxy toluene (BHT)	0.02
Antifungus[c]	0.25
cellulose	0.5
Proximate composition (g kg^{-1})	
Crude protein	40.84
Crude lipid	17.77
carbohydrate	19.53

[a]One kilogram mineral supplementation contented: Co, 100 mg; Cu, 600 mg; Fe, 6 g; Se, 20 mg; Mn, 5 g; Zn, 10 mg; I, 400 mg; choline chloride, 60 g

[b]Vitamin mixture according to Dabrowski et al. (2004)

[c]Antifungi: Toxiban premix (component: aluminosilicate, zeolite, bentonate, propionate ammonium)

400 and 800 mg/kg diet). Briefly, feed ingredients were ground into powder with multi-function pulvetizer, and then were passed through a 1.0-mm sieve. Grounded ingredients were mixed well with the OTC, AA, and oils, and then distilled water was added into the mixture to form a stiff dough. The dough was mixed for 30 min and passed through grinder, dried for 48 h at 25 °C, and finally stored at − 18 °C until use (Kim et al. 2017). Because it was floating diet, feeding was done slowly to make sure the experimental diet was eaten immediately by the fish, and the OTC and AA were not released to the water before eating by fish.

Experimental design

After acclimatization, a total number of 150 rainbow trout were randomly distributed into five experimental groups (A, B, C, D, and E) in triplicates (10 fish per tank) and maintained throughout an experimental period of 28 days. The diet used for all groups (B, C, D, and E) were contained OTC (100 mg/kg body weight) except group A (control). The diet used for groups A and B were supplemented by the normal dose of AA (100 mg/kg feed), while the diets used for groups C, D, and E were prepared by supplementing different high doses of AA (200, 400, and 800 mg/kg, respectively). During the experiment, *O. mikiss* were fed with one of these diets at 2% of biomass and three times (8:00, 13:00, and 19:00) daily.

Sampling

After 28 days of feeding, three fish were randomly harvested from each replicate and immediately anesthetized with powdered clove (200 ppm). Blood samples were drawn from the lateral tail vein using sterile syringes and become two parts. One part was kept in tubes containing heparin for analysis of blood parameters and the second sample was centrifuged at $1500 \times g$ for 5 min to separate plasma (supernatant). The plasma was removed and maintained at − 70 °C until analysis (Yangthong et al. 2016).

Skin mucus was collected according to Shaluei et al. (2017). Briefly, three fish were randomly harvested from each replicate and anaesthetized using clove powder. Each harvested fish was transferred into a separate plastic bag containing 10 ml NaCl (50 mM). The bag was shaken well for 1 min and the skin mucus was immediately poured into 15-ml sterile tubes and centrifuged at $1500 \times g$ for 4 min. The upper layer was collected and maintained at − 80 °C until use.

Analyses

Measurement of blood parameters

Total number of erythrocytes (RBC) and leucocytes (WBC) were determined using a Neubauer chamber by dilution of blood samples in the Natt and Herrick solution (Natt and Herrick 1952). Blood hematocrit (Ht) was measured via micro hematocrit centrifuge at $10{,}500 \times g$ for 5 min (Baba et al. 2015). The hemoglobin amount (Hb) was estimated spectrophotometrically at 540 nm using cyanmethemoglobin method (Harikrishnan et al. 2009). The mean cell volume (MCV), the mean cell hemoglobin (MCH), and the mean cell hemoglobin concentration (MCHC) were determined from the total RBC counts, Hb concentrations, and Ht values according to the available formulae (Azaza et al. 2020). Neutrophils, lymphocytes,

and monocyte numbers were quantified by Giemsa-stained blood smears under light microcopy (Safari and Sarkheil 2018).

Plasma and skin mucus immunology

The total protein and albumin contents of plasma samples were assayed using quantitative detection kits (Pars Azmun Co, Iran). Plasma total protein content was measured by Piotrowski's assay at 540 nm. Plasma albumin content was assayed using bromocresol green dye at 630 nm (Mohammadi et al. 2020a, b). Plasma globulin content was measured by subtracting amount of albumin from total protein (Kumar et al. 2005).

The total immunoglobulin content (Total Ig) of plasma and mucus samples was quantified according to Siwicki and Anderson (1993). The sample was mixed with 12% polyethylene glycol, incubated at 25 °C for 2 h, centrifuged at $3000 \times g$ for 15 min, and then the supernatant removed and the remaining protein was measured after its subtraction from total protein content.

Lysozyme activity of plasma and mucus sample was measured according to method of Ellis (1990) using lyophilized *Micrococcus lysodeikticus* as target in phosphate buffer (pH 6.2). One unit of enzymatic activity was equal to the amount of lysozyme causing a decrease in absorbance of 0.001 at 490 nm.

The method of Yano (1992) involving the use of rabbit red blood cells (RaRBC) as substrate was used to determine the alternative complement activity (ACH50) in plasma. The volume of plasma producing 50% hemolysis of RaRBC was monitored spectrophotometrically at 414 nm.

Protease activity in mucus was measured following Guardiola et al. (2014) using the azocasein hydolysis assay. One unit of protease activity was considered as nanogram of the azo-dye released during 60 s at 37 °C.

Plasma antioxidant enzymes

Blood superoxide dismutase (SOD) and catalase (CAT) activities were assayed spectrophotometrically similar to the method of Yonar et al. (2011). SOD activity was measured by checking the amount of enzyme required to prevent the reduction of nitroblue tetrazolium, which determined at 560 nm. The CAT enzyme activity determination was based on measuring the rate constant of hydrogen peroxide decomposition by this enzyme. The conversion of H_2O_2 to H_2O, and O_2 was measured at 240 nm.

Plasma malondialdehyde content (MDA) was measured spectrophotometrically at 548 nm according to Placer et al. (1966) using the thiobarbituric acid reaction. The MDA activity was expressed as the nanomol of enzyme per milliliter of plasma.

Biochemical parameters

The activities of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were measured spectrophotometrically using quantitative analyses kits (Pars Azmun Co, Iran). ALT activity was expressed as the amount of enzyme needed to form one molecule of pyruvate, which is determined at 340 nm. AST activity was defined as the amount of enzyme needed to form one molecule of glutamate, which is determined at 340 nm. ALP activity was expressed as the amount of enzyme needed to make one micromole of p-nitrophenol, which is determined at 405 nm.

Statistical analysis

The normality of acquired data was determined by Kolmogorov–Smirnov test and the differences between the means were examined via one-way analysis of variance (ANOVA), followed by Duncan test. Differences between mean values were considered to be significant when the confidence level was 95% ($P < 0.05$). Results are defined as mean ± standard deviation for each group. All the statistical analyses were done using the SPSS computer software program (Version 24).

Results

Hematological parameters

The hematological parameters of rainbow trout are presented in Table 2. The RBC, WBC, Hb, Hct, and neutrophils values were significantly changed with the incorporation of OTC in the diet of rainbow trout ($P < 0.05$). The highest numbers of RBC and WBC were recorded in group A (100 mg AA without

Table 2 Changes in hematological parameters of rainbow trout fed with experimental diets after 28 days

Parameters	Treatments				
	A	B	C	D	E
RBC (10^6 mm^{-3})	1.44 ± 0.08^{b}	1.17 ± 0.08^{a}	1.18 ± 0.11^{a}	1.29 ± 0.13^{ab}	1.38 ± 0.11^{b}
WBC (10^3 mm^{-3})	4.52 ± 0.13^{c}	3.56 ± 0.06^{a}	3.66 ± 0.18^{a}	3.95 ± 0.17^{b}	4.17 ± 0.12^{b}
Hb (g dl^{-1})	14.36 ± 1.25^{b}	10.60 ± 0.92^{a}	11.16 ± 1.16^{a}	11.40 ± 1.38^{a}	12.48 ± 1.72^{ab}
Hct (%)	43.56 ± 2.48^{c}	33.98 ± 3.34^{a}	35.14 ± 4.83^{ab}	39.04 ± 3.65^{abc}	41.87 ± 3.59^{bc}
MCV (fl)	302.48 ± 1.46	290.93 ± 9.16	296.90 ± 14.61	302.71 ± 1.24	303.23 ± 3.10
MCH (pg)	99.61 ± 3.07^{c}	90.82 ± 1.48^{ab}	94.50 ± 1.66^{bc}	88.25 ± 2.18^{a}	90.13 ± 5.60^{ab}
MCHC (g dl^{-1})	32.93 ± 0.96^{b}	31.23 ± 0.88^{ab}	31.87 ± 1.21^{b}	29.16 ± 0.82^{a}	29.71 ± 1.59^{a}
Neutrophils (%)	32.66 ± 1.53^{b}	24.33 ± 1.53^{a}	28.33 ± 3.51^{ab}	29.33 ± 4.51^{ab}	27.66 ± 3.05^{ab}
Lymphocytes (%)	60.00 ± 1.00	66.00 ± 2.65	63.66 ± 3.21	63.33 ± 5.03	63.00 ± 1.73
Monocytes (%)	7.33 ± 1.15	9.66 ± 1.15	8.00 ± 1.00	7.33 ± 0.58	9.33 ± 2.08

Values presented as mean ± standard deviation (SD). The absence of letters indicates the absence of significant differences between treatments ($P < 0.05$) for $n = 3$; P values are given

A—diet contained 100 mg AA without OTC; B—diet contained 100 mg AA with OTC; C—diet contained 200 mg AA with OTC; D—diet contained 400 mg AA with OTC; E—diet contained 800 mg AA with OTC

OTC) while the group B (100 mg AA with OTC) showed the lowest values. Feeding fish in the E group (800 mg AA with OTC) significantly increased these parameters as compared to the B group ($P < 0.05$). Hemoglobin, hematocrit, and neutrophils values were significantly lower in group B than in group A (control) and the group which were fed with 800 mg/kg diet of AA (group E) recorded significantly increased Hct value as compared to group B ($P < 0.05$). There was no significant alteration in the MCV, lymphocyte and monocyte values between the five groups.

Biochemical enzymes

As shown in Fig. 1, the plasma AST, ALT, and ALP activities were significantly elevated by the addition of OTC to the diet of fish ($P < 0.05$). The highest activities of these enzymes were recorded in the group B. Fish in the group E exhibited significantly decreased ALT and ALP activities compared with those in the group B. Dietary supplementation with different levels of AA did not cause significant changes in the AST activity ($P > 0.05$).

Plasma immune responses

The measurements of the humoral immunity parameters are presented in Table 3. The lysozyme and complement activities were significantly affected with the incorporation of OTC in the diet ($P < 0.05$). The highest values of these indicators were revealed in the group A whereas the lowest were in the group B. Dietary supplementation of AA significantly improved the lysozyme and complement activities in the group D (400 mg AA/kg diet). There were no significant changes in the total immunoglobulin, total protein, albumin, and globulin levels between the study treatments ($P > 0.05$).

Mucus immune responses

The measurements of the mucus immunity parameters are presented in Table 4. The mucus lysozyme, alkaline phosphatase, and protease activities in the group B (diet contained 100 mg AA with OTC) decreased significantly compared to the control group ($P < 0.05$). The lowest activities of lysozyme and protease were observed in group B and dietary supplementation with additional amounts of AA had no significant change for these parameters ($P > 0.05$). The lowest alkaline phosphatase activity was observed in group B. The dietary supplementation with AA significantly improved alkaline phosphatase activity in the group E (800 mg AA/kg diet) ($P < 0.05$). The total immunoglobulin content did not significantly vary among the different study groups ($P > 0.05$).

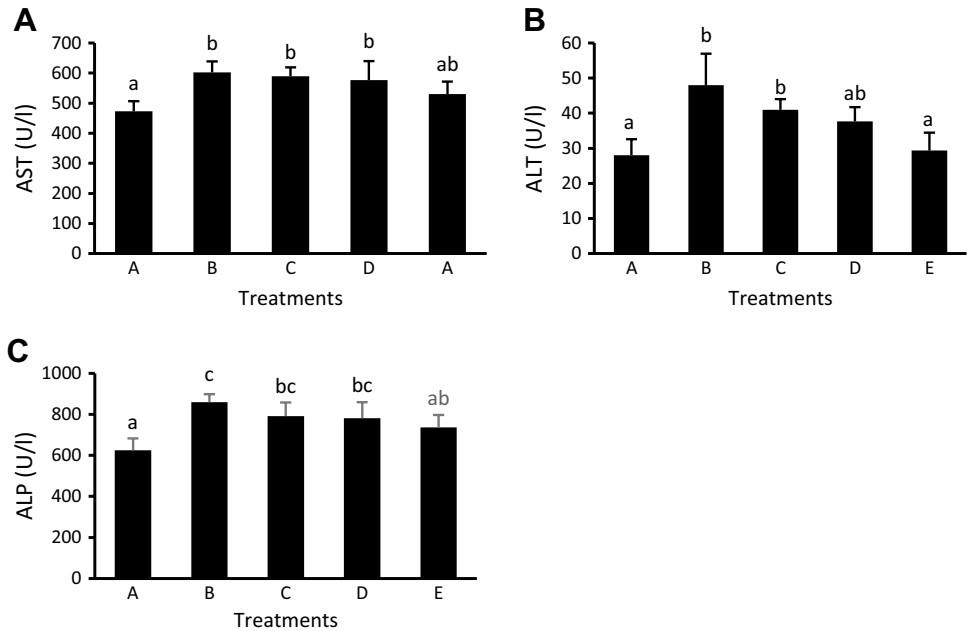

Fig. 1 Activity of AST (**A**), ALT (**B**), and ALP (**C**) in blood of rainbow trout fed with experimental diets after 28 days. The presence of letters indicates significant differences between treatments ($P < 0.05$) for n = 3. A—diet contained 100 mg AA without OTC; B—diet contained 100 mg AA with OTC; C—diet contained 200 mg AA with OTC; D—diet contained 400 mg AA with OTC; E—diet contained 800 mg AA with OTC

Table 3 Plasma immune responses in rainbow trout fed with experimental diets after 28 days

Parameters	Treatments				
	A	B	C	D	E
ACH50 (U ml^{--})	333.02 ± 15.22^{c}	264.64 ± 13.71^{a}	285.15 ± 22.11^{ab}	312.00 ± 26.19^{bc}	293.73 ± 20.42^{ab}
Lysozyme activity (U ml^{-1})	128.43 ± 8.93^{c}	97.42 ± 5.32^{a}	108.25 ± 9.12^{ab}	118.25 ± 13.11^{bc}	113.22 ± 9.75^{abc}
Total Ig (mg ml^{-1})	24.24 ± 2.69	22.04 ± 3.33	22.60 ± 3.09	21.77 ± 2.44	20.48 ± 2.60
Total protein (g dl^{-1})	5.19 ± 0.32	4.74 ± 0.34	4.74 ± 0.68	4.81 ± 0.46	4.71 ± 0.36
Albumin (g dl^{-1})	2.11 ± 0.31	1.86 ± 0.12	1.91 ± 0.31	1.89 ± 0.20	2.01 ± 0.22
Globulins (g dl^{-1})	3.08 ± 0.63	2.88 ± 0.25	2.82 ± 0.51	2.92 ± 0.61	2.70 ± 0.58

Values presented as mean ± standard deviation (SD). The absence of letters indicates the absence of significant differences between treatments ($P < 0.05$) for $n = 3$; P values are given

A—diet contained 100 mg AA without OTC; B—diet contained 100 mg AA with OTC; C—diet contained 200 mg AA with OTC; D—diet contained 400 mg AA with OTC; E^{-}diet contained 800 mg AA with OTC

Antioxidant responses

As seen in Fig. 2, the activities of SOD and CAT were significantly altered by the incorporation of OTC in the diet of fish ($P < 0.05$). The lowest SOD and CAT activities were observed in group B and the groups which were supplemented with higher levels of AA (groups D and E) showed significantly increased SOD and CAT activities. The blood level of MDA found to be significantly increased upon OTC administration in group B compared to control group ($P < 0.05$). The highest activity of MDA was recorded in group B and dietary supplementation with additional levels of AA (200, 400, and 800 mg/kg diet) significantly decreased the level of blood MDA compared to the group B.

Table 4 Mucosal immune responses in rainbow trout fed with experimental diets after 28 days

Parameters	Treatments				
	A	B	C	D	E
Lysozyme activity (U ml^{-1})	54.22 ± 5.53^{b}	43.20 ± 3.24^{a}	45.01 ± 2.50^{a}	48.61 ± 2.50^{ab}	47.56 ± 4.06^{ab}
Total Ig (mg ml^{-1})	10.22 ± 1.40	8.52 ± 1.13	9.74 ± 1.63	8.79 ± 1.43	9.95 ± 1.55
ALP (U ml^{-1})	46.04 ± 1.27^{c}	36.31 ± 3.72^{a}	38.59 ± 5.46^{ab}	38.24 ± 2.64^{ab}	43.37 ± 2.78^{bc}
Protease (U mg^{-1} pr)	40.94 ± 4.97^{b}	27.10 ± 5.62^{a}	29.43 ± 2.21^{a}	28.84 ± 4.03^{a}	32.54 ± 3.10^{a}

Values presented as mean ± standard deviation (SD). The absence of letters indicates the absence of significant differences between treatments ($P < 0.05$) for $n = 3$; P values are given

A—diet contained 100 mg AA without OTC; B—diet contained 100 mg AA with OTC; C—diet contained 200 mg AA with OTC; D—diet contained 400 mg AA with OTC; E—diet contained 800 mg AA with OTC

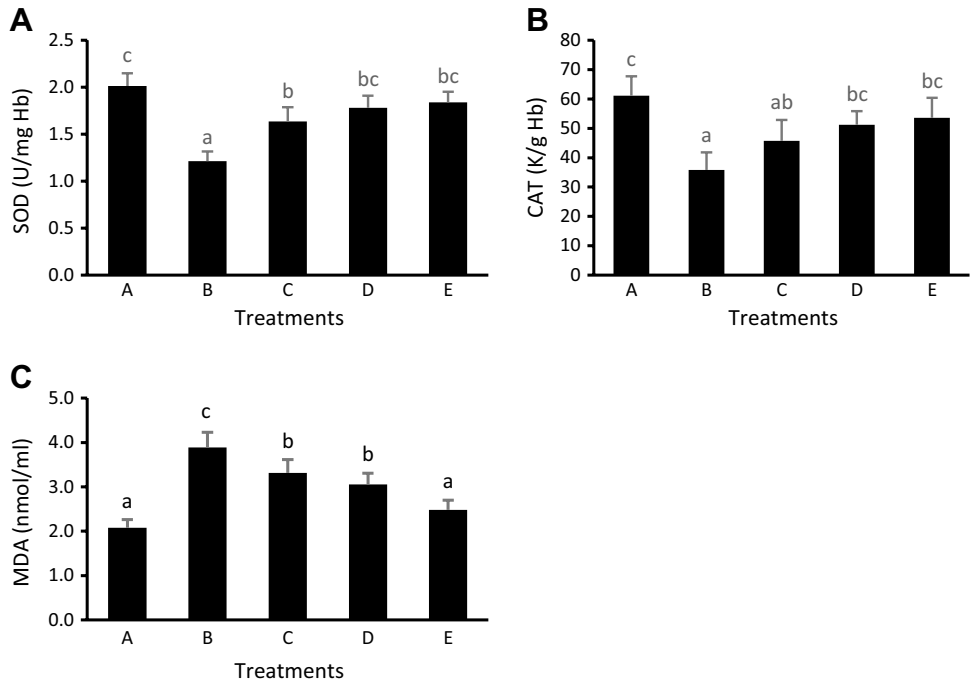

Fig. 2 Activity of SOD (**A**), CAT (**B**) and MDA (**C**) in blood of rainbow trout fed with experimental diets after 28 days. The presence of letters indicates significant differences between treatments ($P < 0.05$) for $n = 3$. A—diet contained 100 mg AA without OTC; B—diet contained 100 mg AA with OTC; C—diet contained 200 mg AA with OTC; D—diet contained 400 mg AA with OTC; E—diet contained 800 mg AA with OTC

Discussion

Vitamin C or L-ascorbic acid (AA) is a water-soluble micronutrient needed for multiple physiological process of aquatic animals (Shahkar et al. 2015). Previous studies showed the anti-stress (Misra et al. 2007), antioxidant (Wan et al. 2014), immuno-stimulatory (Zhou et al. 2012), and growth promoting (Liang et al. 2017) properties of AA in fishes. Considering the beneficial effects of AA, the current study evaluated the possible role of dietary AA in the reduction of OTC-induced stress in *O. mykiss*.

The measurement of hematological indicators is an important tool to monitor the physiological and the pathological alterations in fish (Burgos-Aceves et al. 2019; Javanmardi et al. 2020a, b). In the existing

experiment, the RBC number and the Ht and Hb values were significantly declined by the inclusion of OTC in the diet of *O. mykiss* fed with low dose of AA (100 mg/kg diet). These results suggested that OTC has caused anemia condition in *O. mykiss*, which may be a result of erythropoiesis inhibition and also increased erythrocyte lysis (Ramesh et al. 2018; Yonar et al. 2020). Similarly, significant reductions in RBC, Ht, and Hb values were observed by Omoregie and Oyebanji (2002) in *Oreochromis niloticus* fed diets incorporated with OTC. However, in our study, administration of AA at 800 mg/kg diet markedly restored the RBC and Ht values in fish treated with OTC, which were comparable with control group. This may be due to the anti-oxidative properties of AA which prolongs the life span of erythrocytes by its potent ROS scavenging activity (Nayak et al. 2007). Our finding agrees with the observation of Affonso et al. (2007), who suggested that feeding with higher levels of AA significantly increased the RBC and Ht values in *Brycon amazonicus*.

The study of white blood cell (WBC) profile can provide useful information regarding the general health status of fish. The white blood cells and differential leukocytes number are useful tools to evaluate the immune response in fish (De Pedro et al. 2005; Vali et al. 2020). In this study, OTC administration significantly decreased the WBC and neutrophils values with no significant effect on monocytes and lymphocytes. The reduction in the WBC and neutrophils values may indicate immunosuppressive effects of OTC and subsequently increased fish's susceptibility to infectious diseases. These obtained results are in accordance with the finding of Dobšíková et al. (2013), who observed that OTC decreases the WBC value in *Cyprinus carpio*. On contrary, a significant increase in WBC value was recorded with the supplementation of AA in groups D and E (400 and 800 mg/kg diet respectively) compared to group B, which indicates that the immune system has been restored. Similarly, Misra et al. (2007) suggested that higher levels of dietary AA could induce a significant elevation in WBC count in *Labeo rohita*.

The innate immunity in fish includes two parts of mucosal and humoral immunity which both defense lines play a vital role in the fight against pathogens (Harikrishnan et al. 2012; Hoseinifar et al. 2016). The presence of various factors such as lysozymes, complement, and other lytic components in plasma prevents/or kills microorganisms (Alexander and Ingram 1992). The result of the current study revealed that OTC administration significantly decreased the activities of lysozyme and complement in the plasma of fish fed low dose of AA, suggesting that innate immunity of fish was suppressed following treatment with OTC antibiotic. This OTC-induced immunosuppression is attributable to its high tissue penetration capacity, making OTC capable of interfering with immune system organs, such as the liver and kidney (Yonar et al. 2011). Our results are in accordance with Hoseini and Yousefi (2019) that indicated OTC treatment in rainbow trout significantly decreased lysozyme and complement activities. The results of our experiment also revealed that the plasma lysozyme and complement activities were significantly increased in the D group (fed 400 mg AA/kg diet) compared with those in the group B (fed 100 mg AA per kg diet), which may indicate an improved immune response in fish. In line with our results, several studies showed that dietary AA elevated the innate immune responses in fishes (Li and Lovell, 1985; Hardie et al. 1991; Dunier et al. 1995).

The skin mucus includes various factors such as lysozyme, protease, immunoglobulins, and lectins that play a vital role in the primary defense and protection of fish against pathogens (Hoseinifar et al. 2015; Mohammadi et al. 2020a, b). The results of the present experiment revealed that the mucus lysozyme, protease, and alkaline phosphatase activities markedly decreased with the incorporation of OTC in the diet of rainbow trout fed with low dose of AA (100 mg/kg diet), which may indicate a weakened ability to cope with pathogens. While many experiments have focused on the immunosuppressive effects of OTC on the humoral immunity of fish, these effects in mucosal immunity have not been investigated. Our results also showed that the mucus alkaline phosphatase activity of fish treated with OTC increased significantly in the group E (800 mg AA/kg diet), which may be attributed to an increased mucosal immune response by the AA supplementation at higher doses. Similar to our results, Roosta et al. (2014) observed an increase in the activity of mucus alkaline phosphatase in *Rutilus rutilus caspicus* following dietary supplementation with high levels of AA.

This is well established that increased reactive oxygen species (ROS) levels will lead to oxidative stress, which may negatively affect the fish and

crustaceans' health and cause structural damage in tissue cells (Akbary and Aminikhoei 2018; Tavabe et al. 2020; Khan et al. 2021). Antioxidant defense system includes some pivotal enzymes such as superoxide dismutase (SOD) and catalase (CAT) that tend to prevent ROS formation (Yonar et al. 2014). In our study, a significant decline in the activity of SOD and CAT as well as a significant increase in the level of MDA was observed in the plasma of fish in the group B (100 mg AA with OTC) compared to the control group (100 mg AA without OTC). These recorded changes in SOD and CAT activities can be attributed to an excessive formation of ROS in the fish body, which resulted in the high consumption of these enzymes during elimination of ROS (Rahman et al. 2020). Moreover, the increase in MDA level similarly shows this situation, knowing that MDA is the main product of lipid peroxidation (Yonar 2018). Previously, similar results were obtained from dietary administration of OTC in *O. mykiss* by Nazeri et al. (2017). On the other hand, feeding fish in the D and E treatments (400 and 800 mg/kg diet, respectively) significantly reversed the activity of SOD and CAT enzymes and as well as the MDA level in the fish plasma, indicating the ameliorative effects of dietary AA on OTC-induced oxidative damage. This can be due to the high anti-oxidative capacity of AA which makes it capable of neutralizing ROS and reduction of oxidative stress (Rouhier et al. 2008; Verma et al. 2007). This is in agreement with Wan et al. (2014) who found that higher levels of vitamin C could effectively suppress oxidative stress induced by the high levels of pH.

The measurement of the activity of biochemical enzymes (AST, ALT, and ALP) in the plasma can provide useful information regarding the health condition of fish and crustaceans' liver tissue (Nakano et al. 2018; Javanmardi et al. 2020a, b). In the current study, our results showed significantly increased plasma AST, ALT, and ALP activities in OTC-treated fish (group B) compared to the group A (control). This increase may be linked to the OTC-induced oxidative stress, which affect the permeability of hepatocyte through oxidative damage resulting in leakage of these enzymes to the fish blood (Yonar 2012; Banaee et al. 2011). In line with our findings, the increased activities of biochemical enzymes (AST and ALT) in fish treated with OTC have been previously observed also in *Oncorhynchus kisutch* (Nakano et al. 2018). The results of present study also showed that feeding fish in the group E (diet contained 800 mg AA) significantly decreased these parameters as compared to the B group (diet contained 100 mg AA). These changes may indicate that dietary supplementation with AA helped to reduce the OTC-induced liver damage, resulting in the decreased leakage of these enzymes from liver to the blood. In similar study conducted by Nazeri et al. (2017), feeding with different levels of rutin (a flavonoid) significantly alleviated the activities of plasma enzymes in OTC-treated *Oncorhynchus mykiss*.

In conclusion, the current results showed that the dietary administration of OTC in rainbow trout significantly affected hematological profile, innate immune response, and antioxidant capacity of *O. mykiss*. The low dose of dietary AA was not capable of alleviating the OTC-induced stress. However, supplementation of fish with higher levels of AA found to restore the suppressed immune response as evidenced by increased activities of plasma lysozyme and complement, skin mucus alkaline phosphatase, and augmented WBC and neutrophils values. Moreover, attenuation of OTC-induced oxidative stress by increased activity of anti-oxidative enzymes (SOD and CAT) and decreased activity of plasma biochemical enzymes (AST, ALT and ALP) was also found in fish fed with higher levels of AA. Hence, dietary administration of AA at higher levels could be an effective strategy to decrease the negative effects of OTC on *O. mykiss*.

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Author contribution All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Saeed Moradi, Sina Javanmardi, and Pooria Gholamzadeh. The first draft of the manuscript was written by Saeed Moradi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Supervision and funding acquisition: Kamran Rezaei Tavabe.

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Data availability All data and materials used in the experiment are available and achievable upon request to the corresponding author Sina.javanmardi@ut.ac.ir.

Code availability Not applicable.

Declarations

Ethics approval The trial protocol was approved by the Ethics Committee for the Animal Research, University of Tehran; none of the fish suffered starvation, trauma or electrical shock and all the fish were completely anesthetized before tissue sampling.

Consent to participate All names in author list have been involved in various stages of experimentation or writing and agree with being a part of this work.

Consent for publication All authors agree with submission of the paper for publication in the journal of Fish Physiology and Biochemistry.

Conflicts of interest The authors declare no competing interests.

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