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EFFECTS OF DIETARY VITAMIN A AND E ON GROWTH PERFORMANCE AND ANTIOXIDANT STATUS IN BLOOD OF JUVENILE RAINBOW TROUT (*ONCORHYNCHUS MYKISS,* W. 1792) EXPOSED TO FLOW RATE STRESS

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ABSTRACT

The present study investigated the effects of vitamin A and E supplementation in diets of juvenile rainbow trout subjected to two different flow rates with or without flow stress (0.9 and 2.1 l/min, respectively) on growth performance, vitamin A and E concentrations in serum, malondialdehyde (MDA) level in serum as well as plasma SOD (superoxide dismutase) activity. Fish fed with the three experimental diets (30E+A, 60E+A, –E+A) during 12 weeks. In the unstressed groups, WG (weigth gain) of fish did not differ among the diets groups ($p>0.05$). Lower WG was observed in fish fed the vitamin E-free diet in stressed groups ($p<0.05$). In stress and unstressed groups, SGR (specific growth rate) was not different among all dietary treatments ($p<0.05$). In stressed group, SUR (survivor rate) was highly significant in A+60E and A+30E groups and lower SUR was observed in fishes in the A-E group ($P<0.05$). Both flow rate trials, there was no significant difference in serum vitamin A concentration of all diet groups ($p>0.05$). In the unstressed and stressed groups, serum vitamin E concentration was lowest in fish fed with A-E diets ($p<0.05$). In the unstressed and stressed groups, serum MDA (malondialdehyde) level was highest in fish fed the A-E diet ($p<0.05$). In the unstressed group, SOD activity were similarly affected by 30 and 0 mg/kg vitamin E supplemented diet groups and in both stress conditions plasma SOD (superoxide dismutase) enzyme activity increased in A+60E diet group.

Key words: rainbow trout, stress, growth performance, antioxidan status.

INTRODUCTION

Living organisms are able to adapt to oxidative stress by inducing the synthesis of antioxidant enzymes and damage removal/repair enzymes (Rosa *et al.,* 2005). Like other vertebrates, fish possess an antioxidant system both enzymatic and non-enzymatic. The more relevant antioxidant enzymes consist of glutathione reductase (GRd), superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), and non-enzymatic defenses include vitamin E, vitamin C, vitamin A, coenzyme Q, flavonoids, (Gülçin et al, 2009; Puangkaew *et al.,* 2005). SOD is an important antioxidant enzyme to inhibit oxyradical formation and is usually used as a biomarker to indicate oxidative stress Superoxide dismutase (SOD), a cytosolic enzyme that is specific for scavenging superoxide radicals, is involved in protective mechanisms within tissue injury following oxidative process and phagocytosis. (Jia *et al.,* 2010; Lo´pez-Torres *et al.,* 1993).

On the other hand, some nonenzymatic antioxidants are micronutrients which derive directly from the diet, while others must be appropriately supplied in order to maintain the necessary concentrations in the body (Hidalgo, 2002; Seven *et al.,* 2010). Antioxidant vitamins (mainly A, C and E vitamins) also directly scavenge ROS and upregulate the activities of antioxidant enzymes. These vitamins are among the most important nutrients influencing the organism immune system and their supply can reduce fish mortality and improve performance. Vitamin A (retinol and some retinol metabolites) and pro-vitamin A carotenoids (mainly beta-carotene) are well known dietary antioxidants, respectively, derived from animal and plant sources. (Quiles *et al.,* 2002). Vitamin E has proven beneficial in protecting cellular membranes against oxidation, increases the resistance to stress (Choi *et al*., 2004; Fang *et al.,* 2002; Henrique *et al.,* 1998). High vitamin E content in tissues would inhibit tissue lipid peroxidation (Sau *et al*., 2004). Malondialdehyde is the final product of lipid peroxidation. The concentration of MDA is the direct evidence of toxic processes caused by free radicals (Kandemir *et al.,* 2011; Tatlı Seven *et al*., 2009; Tuna Keleştemur, 2011).

In recent years, there has been a great deal of studies carried out on antioxidant system, but none of these studies investigated effect of the vitamin A and E on biochemical parameters of juvenile rainbow trout under flow rate stress. Thus, this study aimed to investigate the effects of vitamin A and E on growth performance and blood antioxidative status of stressed and unstressed rainbow trout.

MATERIALS AND METHODS

Fish material and experimental desing: Rainbow trout (*Oncorhynchus mykiss*) produced at the Çırçır Hatchery (Elazig, Turkey) were transferred to Keban Dam Lake General Directorate of State Hydraulic Works Laboratory. After the acclimation, fish were selected and randomly stoced. Fish (initial weight and length, 41.43±1 g, 15.41±0.62 cm, respectively) were distributed into 24 fiberglass rectangular (200 cm × 40 cm × 40 cm) tanks with a 4×2×3 experimental design (3 diets group × 2 flow rates × 3 replicate groups) with a density of 20 juvenile rainbow trout (*Oncorhynchus mykiss*) per tank. Experiments were conducted in a tank, supplied with well water two different flow rates, 2.1 and 0.9 l/min respectively. The flow rates that were determined for the low and optimal flow rate groups were stabilized through the use of the water inflow control valves (Tuna Kelestemur and Ozdemir, 2010).

The study was initiated after 1 week-adaptation period of fish to the hypoxic condition at the level of 4.5 mg/l dissolved oxygen. Water temperature was between 8.8 and 9.4 °C and water pH was 8.4 throughout the experiment. The pH of the water in the tanks where the fish were situated was determined by a portable Checker brand pH meter and the dissolved oxygen and the temperature values were recorded through the use of a portable YSI 55 Model 51/12 oxygen probe throughout the duration of the research. Before the fish were anesthesia (15 mg/l Quinaldin), these body weights were measured one every 2 weeks.

Diets: Composition of the basal diet (not supplemented diet of vitamin A and E) is shown in Tablo 1. Experimental diets were formed basal diet with the supplementation vitamin A and E. Three experimental diets (–E+A, 30E+A, 60E+A) were prepared in the laboratory according to the nutritional requirements of rainbow trout. Diet were formulated with the same macronutrient content and considering different levels of vitamin E (Rovimix E-50 adsorbate; min. %50 dl-α-tokopherly acetate) (0, 30 and 60 mg/kg) and vitamin A (Rovimix A 1000; min. 1 000 000 IU per gram, vitamin A asetat) (18 mg/kg). Vitamin A and E were supplied by Turkey DSM nutritional productions firm. Vitamin A and E were not present in the vitamin mixture and were added as a supplemented according to the diet formulation. A small amount of vitamin E in –E diets proved unavoidable since this vitamin was present both in the fish meal and lipid source. Diets were analyzed for vitamin A and E by HPLC. Based on this analysis, level of 13.3, 26.23, 54.41 mg vitamin E/kg diet were determined for –E+A, 30E+A, 60E+A respectively and 14.24, 14.33, 13.83 mg vitamin A/kg diet were determined for, –E+A, 30E+A, 60E+A respectively.

Table 1. Composition and proximate analysis of the experimental diets

Ingredient	Percent of dry weight
Fish (anchovy) meal	50
Soybean meal	23.1
Wheat flour	19.8
Sunflower oil	6
Antioxidant[a]	0.10
Vitamin premix[b]	0.90
Mineral premix[c]	0.10
Proximate compasition (% dry basis)	
Crude protein	43.48
Crude lipid	13.22
Crude ash	4.14

[a]Antioxidant (mg/kg dry diet): Butilen Hydroxytoluene (BHT); 125.000 mg/ kg

[b] Vitamin premix (IU or mg/kg dry diet): Menadion 3.000, Riboflavin 6.000, Pridoksin 5.000, Kobalamin 15, Askorbik asit 150.000, Niasin 25.000, Biotin 40, Folik asit 1.000, Kolin klorid 300, Kalsiyum D-pantothenat 8.000, Kalsiferol 2.000.000 IU.
Mineral premix (mg/kg dry diet): Mn 80.000, Fe 35.000, Zn 50.000, Cu 5.000, I 2.000, Co 400, Se 150.

Ash (650°C, 6 h), crude protein (nitrogen×6.25), ether extract, crude fibre of diets was analyzed by methods of AOAC (1990). The diets were fed to experimental groups of fish satiation (approximately 3% of wet body weigth) per day. Feed wastes and feaces was removed from tanks after feeding. The feeding trial was conducted for 12 weeks.

Blood sampling: At the end of the experiment, blood was obtained from the caudal vasculature of ten fish per tank after the anesthetic process. Serum samples were collected from tubes blood by centrifugation. Plasma samples were collected from heparinized blood by centrifugation. Serum and plasma were stored at -20 °C until analysis (Trenzado, 2007).

Determination of SOD activity levels in plasma and MDA levels in serum: The SOD activity was measured using the kit (RANDOX Laboratories Ltd., Ardmore, Diamond Road, Crumlin, Co. Antrim, United Kingdom). The role of SOD is to accelerate the dismutation of the toxic superoxide radical, produced during oxidative energy processes, to hydrogen peroxide and molecular oxygen. The reaction was based on its inhibitory effect on the rate of superoxide-dependent reduction of nitroblue tetrazolium (NBT) by xanthine–xanthine oxidase that was monitored at 505 nm with a spectrophotometer (McCord and Fridovich, 1969). Serum samples were transferred into polyethylene tubes and 0.5 ml $HClO_4$ (0.5 M), 4.5 ml distilled water and 100 ml-500 ppm 2(6)-di-tert-butyl-p-cresol (BTH) was added to the tubes. Then, the samples were centrifuged at 3500 rpm for 5 min and the supernatants were injected into the HPLC. The mobile phase was 30 mM KH_2PO_4-methanol (82.5+17.5, v/v %,

pH 3.6) mixture and the flow rate was 1.2 ml min^{-1}. The chromatograms were detected at 250 nm and the injection volume was 20 ml (Karatepe, 2004).

Determination of vitamin A and E levels in serum: Serum samples were transferred into polyethylene tubes and 2 ml of ethanol was added to the tubes. Following the addition of 0.3 ml n-hexane that is required for the vitamin extractions into the tubes, they were centrifuged. This step was repeated for two times. N-hexane in the tubes was evaporated using nitrogen. Then the residues were dissolved in the mobile phase (methanol: acetonitrile: chloroform; 47:42:11,v/v/v). The chromatographs were monitored at 326 and 296 nm for vitamins A and E, respectively and the injection volume was set to 50 ml. Techsphere ODS-2 packed column (5 mm particle, 250× 4.6 ID) was used and the flow rate was 1.0 ml min^{-1} (Miller *et al*., 1988).

Statistical methods: All the values were presented as mean±S.E. Differences between group means were assessed by a one-way and two-way analysis of variance (P<0.05, ANOVA) and post-hoc Duncan test used by SPSS/PC (SPSS, 11.5) computer program (SPSS, 1999).

RESULTS

Growth Performance: WG, SGR and SUR values of rainbow trout in 2.1 and 0.9 flow rates at the end of the experiment are presented in Table 2.

Table 2. Effects of dietary supplementation with vitamin A and E on WG, SGR and SUR in juvenile rainbow trout reared during at flow rates of 2.1 and 0.9 l/min.

Experimental Groups	FR[1] (l/min)	WG[2] (g)	SGR[3] (%)	SUR[4] (%)
A+30E	2.1	13.29±1.67*	0.18±0.03*	91.14±2.35*
A+60E	2.1	13.92±2.11*	0.23±0.07*	90.22±3.27*
A-E	2.1	13.08±1.97*	0.18±0.05*	90.16±1.21*
A+30E	0.9	7.18±2.09[a]	0.12±0.02	66.23±1.33[a]
A+60E	0.9	7.75±1.54[a]	0.13±0.02	65.45±2.13[a]
A-E	0.9	5.71±1.89[c]	0.10±0.01	60.17±2.11[b]

Letters indicate significant differences between groups fed with different diets under the same flow rate (P<0.05). *Significant differences between unstressed and stressed for the same group (P<0.05). Each value is mean±S.E. of 3 replicate tanks.
[1] FR: Flow rate
[2]Weigth gain (WG): (final w_t - initial w_t)
[3]Specific growth rate (SGR) (%): [($\log_e$ final w_t - $\log_e$ initial w_t) / duration in days] x 100
[4]Survivor Rate (SUR): 100 x Final number /Initial number

In the unstressed groups, WG of fish did not differ among the diets groups (p>0.05). In stressed groups, fish fed diets with 60 mg E/kg and 30 mg E/kg had highest weight gain, and lowest in fish fed the E-free diet (p<0.05). SGR was not different among all dietary treatments (P>0.05). In stressed group, SUR was highly significant in A+30E and A+60E groups and lower SUR was observed in fishes in the A-E group (P<0.05). Also, it was found that the SUR was not significantly affected by supplemented diets (P>0.05) in unstressed groups (Table 2).

The results of ANOVA test showed that WG, SGR and SUR values of fish were negatively affected by low flow rate (Table 2). Considered separately, diet and FR interactions and FR significantly affected the WG (P<0.05, P<0.05, respectively), however not by diet (P>0.05, Table 4). FR significantly affected the SGR and SUR values (P<0.05, Table 4), but did not effect from diet, diet and FR interactions (p>0.05).

Vitamin A and E levels in serum: Both flow rate trials, there was no significant difference in serum vitamin A concentration of diet groups (p>0.05, Table 2). In the unstressed group serum vitamin E concentration was highest in fish fed the diet with 60 mg E/kg, followed by 30 mg E/kg and lowest in fish fed the diet with vitamin E-free. In the unstressed group, serum vitamin E concentration was lowest in fish fed diets with vitamin E-free.

However, vitamin A and E concentrations in serum were negatively affected by low flow rates in all groups. FR significantly affected serum vitamin A levels (P<0.05), however not by diet, diet and fr interaction (P>0.05, Table 4). The interaction between diet and fr (Table 4) was found to affect significantly the deposition of vitamin E in the blood (P<0.05). Diet and FR significantly affected blood vitamin E concentration (Table 4, P<0.05, P<0.01, recpectively),

MDA levels and SOD actvity in blood: The results of ANOVA test showed that MDA levels of fish tissues were positively affected by dietary supplementation of vitamin E (Table 3). In the unstressed and stressed groups, serum MDA level was highest in fish fed the diet

with vitamin E-free, and lowest in fish fed diets with 60 mg E/kg. FR not affected serum MDA levels of fishes (P>0.05, Table 4). Diet and the interaction between diet and fr significantly affected the MDA levels (P<0.05, Table 4).

Table 3. Effects of dietary supplementation with vitamin A and E on serum vitamin A and E concentration, serum MDA level and plasma SOD enzym activity of rainbow trout at flow rates of 2.1 and 0.9 l/min.

Experimental Groups	FR (l /min)	Serum Vit. A (mg/g)	Serum Vit. E (mg/g)	Serum MDA (nmol/ml)	Plasma SOD (U/ml)
A+30E	2.1	0.61±0.06*	16.23±0.92^{b*}	8.41±2.03^{b}	12.3±0.41^{b*}
A+60E	2.1	0.75±0.06*	22.42±1.44^{a*}	5.16±1.12^{c}	13.1±0.17^{a*}
A-E	2.1	0.63±0.01*	10.03±1.31^{c*}	14.28±3.21^{a}	11.7±0.35^{b*}
A+30E	0.9	0.26±0.04	9.45±0.44^{a}	13.25±2.13^{b}	6.9±0.11^{b}
A+60E	0.9	0.37±0.06	8.05±0.54^{a}	9.12±1.82^{c}	7.4±0.17^{a}
A-E	0.9	0.21±0.06	5.17±0.30^{b}	18.21±3.14^{a}	6.2±0.35^{c}

$^{a-c}$Letters indicate significant differences between groups fed with different diets under the same flow rate (P<0.05). *Significant differences between unstressed and stressed for the same group (P<0.05). Each value is mean±S.E. of 3 replicate tanks.

Table 4. Two-way ANOVA showing the effect flow rate (FR), Diet and FR×Diet interaction on plasma SOD (superoxidase dismutase) enzym activity, serum MDA (malodyaldehyde), serum vitamin A and E concentrations, weigth gain (WG), specific growth rate (SGR) and survivor rate (SUR).

	FR	Diet	FR×Diet
Plasma SOD (U/ml)	0.01	0.05	0.05
Serum MDA (nmol/ml)	ns	0.05	0.05
Serum Vit. A (mg/g)	0.05	ns	ns
Serum Vit. E (mg/g)	0.01	0.05	0.05
WG (g)	0.05	ns	0.05
SGR (%)	0.05	ns	ns
SUR (%)	0.05	ns	ns

ns: no significant differences (P>0.05).

In the stressed group, plasma SOD enzym actvity were highest in fish fed diets with 60 mg E/kg, followed by 30 mg E/kg, and lowest in fish fed the E-free diet (P<0.05, Table 2). In the unstressed group, SOD enzym actvity were similarly affected by 30 mg/kg vitamin E supplemented diet and vitamin E–free diet (Table 2). SOD enzym activity significantly decreased in these diet groups compared to 60 mg/kg vitamin E supplemented diet (P<0.05). In the stressed group had lower plasma SOD enzym acvtivity than in the unstressed group irrespective of the vitamin E supplementation levels (Table 3). FR significantly affected plasma SOD enzym acvtivity (P<0.01, Table 4). Diet and the interaction between diet and fr significantly affected plasma SOD enzym acvtivity (P<0.05, Table 4).

DISCUSSION

Reductions of oxygen concentration in water have resulted in significant changes in antioxidant defense system in teleosts. Occured stress leads to generation of free radicals, such as O_2^- and HO (Lushchak and Bagnyukova, 2006). These free radicals can damage cell membranes by inducing lipid peroxidation of polyunsaturated fatty acids in the cell membrane (Sorg, 2004). Vitamin E (α-tocopherol) function as biological antioxidants to protect cellular macromolecules (DNA, protein, lipids) and other antioxidant molecules from uncontrolled oxidation by free radicals during normal metabolism or under the conditions of oxidative challenge such as infection, stress, and pollution (Huang and Huang, 2004). Survival of fish fed the vitamin E supplemented diets was higher in general. Vitamin E functions as a lipid-soluble antioxidant protecting biological membranes and lipoproteins against oxidation, and it has been demonstrated to be an essential dietary nutrient for all fish studied (NRC, 1993). Vitamin A occurs in three forms: the alcohol (retinol), aldehyde (retinal) and acid (retinoic acid) in animal tissues. The main physiological functions of vitamin A are differentiation of epithelial tissues, resistance to infections, proper growth, reproduction and vision. (Mohamed *et al.,* 2003). Vitamin A is recognised as a key factor in embryonic development through the regulation of cell differentiation and proliferation, and for its effects in vision growth, stress and normal function of the immune system. For freshwater fish, the requirement has been estimated to be from 600 to 1200 ug retinol kg^{-1} diet to maintain growth (Hemre *et al.,* 2004). In the stressed and unstressed group, WG deteriorated in trout fed the only vitamin A supplemented diet as compared to the fish fed vitamin A and E participate with diets. In our study, two antioxidants (vitamin A and E) significantly decreased negative effects of low flow stress on WG and SUR. These antioxidants also increased WG and SGR values of fishes under the unstressed conditions.

High vitamin E content in tissues would inhibit tissue lipid peroxidation. Similar trends have been

reported in rainbow trout, turbot, Atlantic salmon, sea bass, and Atlantic halibut (Sau et al., 2004). Huang and Huang (2004) determined that WG of the juvenile hybrid tilapia (*Oreochromis niloticus* × *O. aureus)* fed diets containing 0 IU vitamin E/kg was significantly lower ($P < 0.05$) than those fed higher vitamin E (>80 IU/kg) diets and improved in SUR. They also determined that both liver and muscle vitamin E contents increased when dietary vitamin E level increased and lipid peroxidation in tilapia tissues was significantly influenced by the dietary vitamin E level ($P < 0.05$). Results from the present study are in consistent with previous studies where the dietary supplementation of vitamin E at elevated levels increased the concentration of tocopherol and decreased lipid peroxidation in blood of fish. Lin and Shiau (2005), reported that dietary vitamin E supplementation increased growth performance and decreased MDA levels in grouper. Tuna Keleştemur *et al.* (2012) reported that antioxidant vitamin concantrations (A,E and C) decreased in serum MDA level. Similar findigs were obtained in our study. Additionally, only vitamin A supplemented diet was not enough to suppress lipid peroxidation compared to vitamin E supplementation.

Obviously, SOD is an important enzyme family in living cells for maintaining normal physiological conditions and coping with stress (Chan and Pan, 1996). Antioxidant defenses in fish are depend on feeding behavior and nutritional factors. In addition, environmental conditions and seasonal changes have been reported to influence antioxidant defenses of fish (Rosa *et al.,* 2005). Palace *et al.* (1993) observed that SOD activity in the tissues of rainbow trout fed with α-tocopherol deficient diet increased. Similar results were also reported in hybrid tilapia (Huang and Huang, 2004) fresh water pawn (Dandapat *et al.,* 2000). In our study was observed that the addition of 60 mg/kg vitamin E significantly decreased the plasma SOD activity compare to the other groups at optimal flow rate, but significant differences were not observed the plasma SOD activities of fish exposed to low flow rate treatment.

SOD activity of 60 mg vitamin E added group was lower compared to other groups at 2.1 l/min flow rate treatment (optimal flow rate). However, some studies have reported a decrease (Wohaieb and Godin, 1987; Ozkaya *et al.,* 2002) while others have reported an increase in SOD activity in case of lipid peroxidation such as SOD in lipid peroxidation (Aliciguzel *et al.,* 2003; Huang *et al.,* 1999). In a study where the effect of caffeic acid were investigated on antioxidant enzym activity in rats exposed to cold stres. It was determined that cold stress significantly decreased SOD antioxidant enzym activity (Ates *et al*., 2006). Similary, in our study, it was determined that the plasma SOD activity significantly decreased in juvenile rainbow trout exposed to stress.

In conclusion, dietary supplementations (together supplementary vitamin A and E) of juvenile rainbow trout with antioxidants alleviated the flow stress-induced oxidative damages. Two antioxidants together effects significantly decreased the negative effects of flow stress on WG, SGR and SUR. These positive effects were supported by increasing growth performances and SUR of fishes fed on supplemented diets under low flow stress conditions. Moreover, vitamin E is more effective on WG and SUR values of stressed trout, while vitamin E supplementation (60 mg/kg diet) is more effective in preventing lipid peroxidation in the blood and it also improves vitamin E concentration in the blood. The antioxidant status deteriorated in trout fed the only vitamin A supplemented diet as compared to the fish fed vitamin A and E together supplemented diets. Additionally, vitamin E supplementation also increased the SOD enzym activity in plasma of stressed and unstressed juvenile rainbow trout.

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