Growth performance, hematology and immunological parameters of rainbow trout, *Oncorhynchus mykiss*, fed with diets containing different levels of vitamin E and folic acid

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Abstract

This study was conducted to evaluate the effects of dietary levels of vitamins E (25, 30 and 35 mg kg^{-1}), Folic Acid (1.5, 2 and 2.5 mg kg^{-1}) and their combination on the growth performance, hematological and immunological parameters of rainbow trout, *Oncorhynchus mykiss*. Each diet was fed to rainbow trout in triplicate to apparent satiation four times daily for 8 weeks. At the end of the feeding trial, the final body weight (FBW), total length (TL), feed conversion ratio (FCR), specific growth rate (SGR) and survival rate were significantly affected by treatments and the fish fed with the diet containing 35 mg kg^{-1} vitamin E plus 2.5 mg kg^{-1} of folic acid had higher values than those fish fed with the other diets. The results also indicated that hematocrit (Ht), mean corpuscular volume (MCV) mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were significantly influenced by the treatments. Lysozyme and immunoglobulin (IgM) concentrations were significantly influenced by the dietary treatments, fish fed with the diet supplemented with 30 mg kg^{-1} of vitamin E plus 2 mg kg^{-1} of folic acid and unsupplemented diet had higher concentrations than those fish fed with the other diets. The findings of this study suggested that trout requires 35 mg kg^{-1} vitamin E and 2.5 mg kg^{-1} folic acid for it's normal growth and physiology.

Keywords: Vitamin E, Folic acid, Rainbow trout, Hematology, Non-specific immunity

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Introduction

Vitamins are organic substances that are necessary for health growth and maintenance. Vitamin E activity is present in a group of naturally occurring closely related tocopherol. Among them, α-tocopherol has the highest vitamin E activity. DL-α-tocopherol acetate, a stable vitamin of α-tocopherol, is the most commonly used form in animal feeds (NRC, 1993). It is required for all vertebrate animals, and it is an indispensable micro-nutrient which plays important roles in various biochemical and physiological processes, including improving growth performance (Kocabas and Gatlin, 1999), enhancing immunity (Trushenski and Kohler, 2007; Verlhac Trichet, 2010). It has been well demonstrated that the deficiency of some micronutrients produces pathological symptoms and, thus, immunodepression. A dietary requirement of vitamin E has been recognized in a number of fish. Most of the deficiency signs are observed in fish, such as fatty liver degeneration, anemia, erythrocyte hemolysis, hemorrhage, and reduction of fertility (NRC, 1993). Folic Acid is also an essential nutrient for fish (Halver, 1989), as it is for other vertebrates. A requirement was demonstrated in rainbow trout and other salmonids by (McLaren *et al*., 1947; Halver, 1957). Deficiency of folic acid is consistently resulted in megaloblastic anemia together with anorexia and associated low weight gain (WG). The hematological changes occurring in folic acid-deficient Coho salmon, *O. kisutch* have been documented by Smith (1968) and Smith and Halver (1969). In most studies, biological effects of nutrients are often evaluated only in isolation. There are clear evidences that nutrients do not function as independent units and are interrelated with other nutrients in terms of function and metabolism (Hilton, 1989). Thus nutrient level of a particular diet might affect by the level of another nutrient in either the diet or metabolically in the animal. Also exogenous dietary nutrients play important role and recent studies suggest that components can interact to spare or replace each other (Bell *et al*., 2000). Thus, this study was conducted to evaluate the effect of dietary levels of vitamins E and folic acid and their combination on the growth performance, hematological and immunological parameters of rainbow trout, *O. mykiss*.

Materials and methods

Experimental diets preparation

A basal practical diet was formulated to contain approximately 42% crude protein, 12% lipid and 16.8 MJ/kg gross energy, based on the feedstuff values reported in National Research Council (NRC, 1993) (Table 1). The basal diet was supplemented with three levels of vitamin E as a-tocopheryl-acetate with 250 IU activity g^{-1} (25, 30 and 35 mg kg^{-1} diet) and three levels of folic acid as 98% folic acid concentrate as a folate source to prepare nine experimental diets as follow: T_1: 25 mg of vitamin E, T_2: 30mg of vitamin E, T_3: 35mg of vitamin E, T_4: 1.5mg of folic acid, T_5: 2mg of folic acid, T_6: 2.5mg of folic

acid, T_7: 25mg of vitamin E + 1.5mg of folic acid, T_8: 30mg of vitamin E+2mg of folic acid and T_9: 35mg of vitamin E +2.5 mg of folic acid equivalent kg^{-1} diet. Also, a treatment without addition of vitamin E and folic acid was used as control. The ingredients were homogenized by a mill and oil and water were then added. After homogenization, diets were pelleted and then dried in a forced-air oven at 55°C for 24h. Pellets were broken to sizes appropriate for the animals. A pellet crusher was used to adjust the granule size.

Table 1: Composition of the experimental diet (dry weight).

Ingredients (g/100 g as is basis)	**Percent in diet**
Fish meal	42
Wheat flour	15
Soybean meal	25
Fish oil	11.5
Vitamin mixture (Vitamin E free)[a]	2.5
Mineral mixture [b]	1.5
Salt	2
Proximate composition	%
Crude protein	49.18
Crude lipid	14.12
Moisture	14.25
Ash	20.70
Gross energy(MJ/kg)	16.8

[a] Vitamin mixture was manually provided according to feed requirements of the fish and ingredients were obtained from Science Laboratories (Ghazvin, Iran); which each 1000 g vitamin mixture provides vitamin A, 1,600,000 I.U; vitamin D3, 400 000 I.U; thiamin, 6 g; riboflavin, 8 g; niacin, 12 g; pantothenic acid, 40 g; pyridoxine, 4 g; folic acid, 2 g; cyanocobalamin, 8 mg; vitamin C, 60 g; vitamin K3, 2 g; biotin, 240 mg and inositol, 20 g.

[b]Mineral premix (mg kg) 1 diet: NaCl, 500; $MgSO_4.7H_2O$, 7500; $NaH_2PO_4.2H_2O$, 12 500; KH_2PO_4, 15 500; $Ca(H_2PO_4)2H_2O$, 10 000; $(CH_2CHCOO)2Ca.5H_2O$, 1650; $ZnSO_4.7H_2O$, 176.5; $MnSO_4.4H_2O$, 81; $CuSO_4.5H_2O$, 16.5; $CoCl_2.6H_2O$, 0.53; KI, 1.59; starch, 147.5.

Experimental fish and feeding trial

A total of 990 rainbow trout fingerlings (average weight: 8.11±0.11g; mean±SD) stocked into 30 fiberglass tanks (100L) at Cold Water Fish Research Centre at Tonekabon (Mazandran, Iran). There were three replicates per treatment. Fish were fed at 2% body weight with adjustments made in the quantity of feed supplied every week. Feeding was done four times a day. Feeding was carried out for 8 weeks. A diurnal 12-h light: dark cycle was provided by fluorescent lights. Total fish weight in each tank was measured every 2 weeks for more accurate feeding rate adjustment. Dissolved oxygen, water temperature and pH of the water were monitored throughout the experiment.

Growth performance

At the end of the feeding trial, fish were fasted for 24 h and then weighed, TL, WG, FW, FCR, SGR, CF and survival rate of rainbow trout were calculated according to Huang *et al*. (2003).

FCR = dry feed intake (g)/wet WG (g)

SGR (% day^{-1}) = (Ln W_f - Ln W_i) × 100/t

CF = 100×[wet weight (g)/TL $(cm)^3$]

Where W_f and W_i were final and initial fish weights, respectively; TL was total length and t is the experimental duration in days.

Haematological assay

Haematological parameters were evaluated by randomly removing fish after 60 days. Fish were anaesthetized by clove powder at 100 ppm in water and then blood samples were collected via venipuncture and aspirated into a microcentrifuge tube. The first sample was transferred to an eppendorf tube coated with heparin as anticoagulant and was used for hematological indices determination including Ht, number of red blood cell (RBC) and white blood cell (WBC). RBC($x10^6$) and WBC were determined with a Neubauer using Rees diluting solution. To determine differential counts of leukocyte, that is measure of lymphocyte, neutrophil, eosinophil and monocyte, the obtained smears were first air dried, fixed in 96% ethanol for 30 min, stained by Giemsa staining for 30 min and were examined for leukocyte differential count under light microscope (Klontz, 1994). Hemoglobin concentration (Hb) was determined with Drabkin's reagent and read the absorbance at 540 nm (Jain, 1993). According to the procedure of Rehulka (2000), Ht was measured in microhaematocrit heparinised capillaries, using a microhematocrit centrifuge (13,000 rpm for 3 min). MCV and MCH were obtained according to the method described by Haney *et al*. (1992). Blood was centrifuged at 3000 rpm for 15 min in cooling centrifuge for separation of plasma which was stored at -18 ºC till used for biochemical analysis. Lysozyme level was determined by turbidometric assay according to the method of Sankaran and Gurnani (1972) with slight modifications. Aliquots (1.75 mL^{-1}) of *Micrococcus lysodeikticus* suspension (Sigma) (0.375 mg mL^{-1}, 0.05 M PBS, pH 6.2) were mixed with 250 ml^{-1} of each sample and the optical density was measured after 15 and 180 s by spectrophotometer (Biophotometer Eppendorf) at 670 nm. PBS was used as the blank and results were expressed in amounts of lysozyme (mg) per 1 mg of sample calibrated using a standard curve determined with hen eggs white lysozyme (Sigma) in sterile sodium phosphate buffer. The IgM content was determined following the method of Puangkaew *et al*. (2004).

Statistical analyses

Data were analyzed by one-way analysis of variance (ANOVA) to test the effects of dietary levels of vitamins E, folic acid and their combination. Differences were considered to be significant at the 0.05 probability level. All analyses were performed using SPSS software (version 17). All assays were carried out in

triplicates and data are shown as mean±SD for each dietary group.

Results

The average growth parameters at the end of the trial are given in Table 2. Most growth parameters including FBW, TL, FCR and SGR were influenced by diets containing various levels of vitamin E, Folic Acid and their combination, and higher values were observed in the diet containing 35 mg/kg-1 vitamin E+2.5 mg Folic Acid mg/kg-1 (T9) (p<0.05). The best FCR was attained in diet supplemented with 30 mg/kg-1 vitamin E +2 mg folic acid mg/kg-1 (T8). Survival at the end of week 8 was affected by supplementation of vitamin E, folic acid and their combination (p<0.05). The hematological characteristics are presented in Tables 3 and 4, respectively. Significant difference was observed in hematological parameters including Ht, MCV, MCH and MCHC among dietary treatments. Fish fed with the basal diet had lower Ht value than those fed with the other diets (p<0.05). IgM concentration and plasma lysozyme activity were significantly influenced by the dietary vitamin E and folic acid levels (Fig. 1 and 2), fish fed with the basal diet had significantly higher values than those fed with other diets (p<0.05).

Table 2: Final body weight (FBW), weight gain (WG) (g/fish), total length (TL), feed conversion ratio (FCR), specific growth rate (SGR) and condition factor (CF) of rainbow trout fed with the experimental diets for 8 weeks.

Treatments	Initial weight (g)	Final weight (g)	TL (cm)	FCR	SGR (% day^{-1})	Condition factor	Survival rate (%)
Control	8.12±0.02	24.8±8.3 ^{c}	14.4±0.53 ^{b}	1.5±0.06 ^{d}	2.07±0.05 ^{bc}	0.91±0.04	85.5±1.9 ^{a}
T_1	8.13±0.02	24.5±8.1 ^{d}	14.8 ±0.61 ^{c}	1.4±0.04 ^{cd}	2.06±0.03 ^{abc}	0.84±0.07	95.5±1.9 ^{cd}
T_2	8.14±0.01	25.7±8.3 ^{e}	15.2±0.62 ^{d}	1.4±0.014 ^{cd}	2.12±0.03 ^{bcd}	0.76±0.00	97.7±1.8 ^{d}
T_3	8.12±0.06	30±5.8 ^{f}	16.1 ±0.23 ^{g}	1.2±0.02 ^{b}	2.31±0.02 ^{cd}	0.89±0.04	95.5±1.7 ^{cd}
T_4	8.15±0.02	23.3± 4.3 ^{a}	14.4±0.51 ^{b}	1.4±0.07 ^{cd}	2±0.06 ^{ab}	0.78±0.01	93.3±0.00 ^{c}
T_5	8.10±0.05	28.2 ± 6.1 ^{g}	15.8±0.50 ^{f}	1.2±0.06 ^{b}	2.23±0.06 ^{bcd}	0.90±0.02	97.7±1.5 ^{d}
T_6	8.11±0.04	27±7.1 ^{ef}	15.5±0.45 ^{e}	1.2±0.04 ^{b}	2.18±0.05 ^{bcd}	0.94±0.00	92.2±1.6 ^{bc}
T_7	8.12±0.03	23.9±7.8 ^{d}	14.4 ±0.48 ^{b}	1.4±0.04 ^{cd}	2.03±0.03 ^{ab}	0.75±0.00	96.6±0.00 ^{d}
T_8	8.13±0.02	22.1±5.7 ^{d}	13.9±0.29 ^{a}	1.3±0.05 ^{d}	1.94±0.03 ^{a}	0.91±0.04	94.4±3.8 ^{cd}
T_9	8.13±0.01	32.1±10.5^{d}	16.3±0.36 ^{g}	0.9±0.06 ^{a}	2.36±0.08 ^{d}	0.96±0.07	97.7±2.2 ^{e}

Within a column, means with different superscripts are significantly different (p< 0.05)

Table 3: Hematological parameters of rainbow trout fingerlings fed 8 weeks with diets containing different levels of vitamin E and folic acid.

Treatments	RBC ($\times 10^6$)	WBC ($\times 10^3$)	Hematocrit (%)	Hemoglobin (gr/dl)	MCV (fl)	MCH (pg)	MCHC (gr/dl)
Control	121±17.3	76.6±17.8	36±2.6 ^{bcd}	6.3±0.48	299±21.4 ^{cde}	52.6±6.3 ^{a}	17.5±1.6 ^{a}
T_1	136±28.1	58.6±15.2	30.6±3.5 ^{ab}	7.2±0.25	228±23.4 ^{a}	54.2±8.7 ^{ab}	23.7±2.2 ^{c}
T_2	147±17.5	52±14.4	34±3 ^{abc}	7.5±0.74	231.3±7.10 ^{a}	51.4±1.02 ^{a}	22.2±0.02 ^{ab}
T_3	121±0.10	70.6±10.2	31.6±3.05 ^{ab}	7.2±1.08	260±24.5 ^{abc}	59.8±7.10 ^{ab}	22.9±1.1 ^{ab}
T_4	110±12.7	87.6±11.6	30.3±1.5 ^{a}	7.2±0.46	274.8±12.4 ^{bcd}	65.8±3.9 ^{bc}	23.9±1.5 ^{c}
T_5	112±15.7	86±11	31±4.3 ^{ab}	6.9±0.66	276±3.2 ^{e}	62.5±3.4 ^{abc}	22.6±1.3 ^{ab}
T_6	124±13.9	78.3±12.2	40.6±2.3 ^{d}	8.9±0.67	327.2±12.7 ^{ab}	72.02±6.1 ^{c}	21.9±0.43 ^{ab}
T_7	139±16	76.6±9.2	34.6±2.5 ^{abc}	7.4±0.12	250.2±34.2 ^{de}	53.4±3.9 ^{a}	21.4±1.3 ^{ab}
T_8	113±10.3	75.3±7.5	35.6±0.57 ^{abcd}	7.4±0.14	315.1±17.2 ^{bcd}	65.7±2.1 ^{bc}	20.8±0.07 ^{b}
T_9	133±17.3	76.6±13.7	37±2.6 ^{cd}	7.5±1.15	279.2±36.7 ^{bcd}	57.3±10.9 ^{ab}	20.4±1.7 ^{b}

Within a column, means with different superscripts are significantly different ($p< 0.05$).

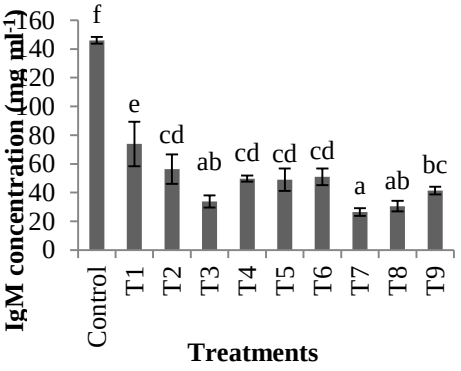

Figure 1: IgM concentration of rainbow trout fingerlings fed 8 weeks with diets containing different levels of vitamin E and folic acid.

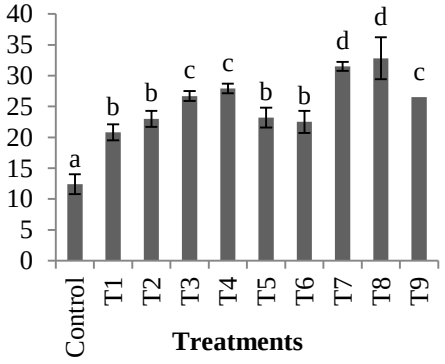

Figure 2: Lysozyme activity of rainbow trout fingerlings fed 8 weeks with diets containing different levels of vitamin E and folic acid.

Discussion

The requirement for vitamin E as an essential dietary component in fish has long been recognized. According to literature, vitamin E deficiency signs such as retarded growth (Peng and Gatlin, 2009), darkened skin, hemorrhaging (Chen *et al*., 2004) and low hematocrit (Kocabas and Gatlin, 1999). Significant effects of dietary vitamin E, Folic Acid and their combination on survival or growth of the fish were observed in the present trial.

Also similar results have been found for hybrid striped bass, *Morone chrysops* ×*M. saxatilis* (Kocabas and

Gatlin, 1999), Rohu, *Labeo rohita* (Sahoo and Mukherjee, 2002) and mrigal, *Cirrhinus mrigala* (Paul *et al*., 2004). But, growth was not affected by dietary a-tocopheryl acetate inclusion in diets for channel catfish, *Ictalurus punctatus* (Bai and Gatlin, 1993), African, *Clarius gariepinus* (Baker and Davies, 1996) and rainbow trout, *O. mykiss* (Boggio *et al*., 1985; Kiron *et al*., 2004). Also, Lygren *et al.* (2000) reported no difference in SGR of Atlantic salmon (*Salmo salar*) fed with three different levels of dietary vitamin E. On the other hand, Amago salmon (*O.rhodurus*) fed with a diet without vitamin E supplementation showed decrease in body weight although survival rate was not significantly different from that of control fish (Taveekijakarn *et al*., 1996). Differences in the individual size, development stage, cultivation environment and variation in experimental conditions including levels of interacting nutrients in the experimental diets may have resulted in the discrepancies observed in this trial compared with other studies. It has been demonstrated that smaller fish implies higher vitamin E requirements, and the more HUFA in diets the more vitamin E is required (Baker and Davies, 1996). Previous studies have shown that fish fed folic acid-deficient diets exhibited growth retardation, anemia, reduction of hematocrit and hemoglobin (Smith and Halver 1969; Duncan *et al*., 1993; Shafaeipour *et al*., 2011). Although, there is not enough literature evaluating dietary of folic acid for fish with haematological assays, there are few results regarding its effects on growth performance. Mahajan and John (1979) reported compromised growth performance in Milkfish, *Channa punctatus* fed diets without folic acid. This was also described by Duncan and Lovell (1994) for channel catfish. *Penaeus monodon* prawns showed declines in the weight gain, feed efficiency and survival rate in low folic acid levels (Shiau and Huang 2001a). Shiau and Huang (2001b) showed that folic acid was essential for growth performance in hybrid tilapia, *O.niloticus* × *O. aureus*, determining the value to be 0.82 mg folic acid kg^{-1} diet. Lim and Klesius (2001) also demonstrated values ranging from 0.5 to1.0 mg folic acid kg^{-1} diet are sufficient for normal development and haematology in Nile tilapia. Inclusion of vitamin E and folic acid in diet resulted in change of some hematological parameters in rainbow trout. As it is shown in Table 4, Ht, MCV, MCH and MCHC were higher in fish fed with different levels of vitamin E and folic acid than those fish fed with the control diet. Also, no significant trend was observed regarding to the differential among white blood cell counts. Similar to our results, no significant differences were observed in hematological parameters in the number of red blood cells and hemoglobin of Gilthead seabream (Montero *et al*., 2001) and Indian major carp (Sahoo and Mukherjee, 2002). Previous studies in Nile Tilapia (Barros *et al*., 2009) and

beluga, *Huso huso*, demonstrated dietary folic acid-deficient did not significantly influence RBC level. This could be explained by the hypothesis that intestinal micro-organisms may contribute to production of folic in sufficient amounts to maintain an acceptable RBC level. It has been demonstrated that intestinal microorganisms are a significant source of folic acid as reported for Common Carp (Kashiwada *et al*., 1971) and hybrid tilapia (Shiau and Huang 2001b), showing that some species do not need this vitamin supplementation. However, interaction of vitamins E with some other nutrients can achieve significant results in terms of physiological indices. However, there is a lack of literature data on vitamins E and folic interactions on hematological parameters of rainbow trout. It is well recognized that dietary levels of vitamin E enhance immune responses in farmed fish (Hardie *et al*., 1990). Increased levels of vitamin E protected channel catfish (*Ictalurus punctatus*) from disease (Bai and Gatlin, 1993). Naziroglu *et al*., (2003) stated that vitamin E especially a-tocopherol form, plays effective role on immune system response, and it is one of the few nutrients for which supplementation with higher than recommended levels enhance certain aspects of immune function in fish. In the present study, lysozyme activity and IgM concentration of fish fed different levels of vitamin E and folic acid showed significant difference, fish fed the basal diet had significantly higher values than those fed other diets. Dietary vitamin E had no significant effect on lysozyme activity of hybrid Stripped bass (Sealy and Gatlin, 2002) and rainbow trout (Kiron *et al*., 2004). However, Indian major carp fed diets supplemented with vitamin E generally had higher plasma lysozyme activity than in that of fish fed with the unsupplemented control diet (Sahoo and Mukherjee, 2002). One of the reasons for such differences in results is considerable variation in lysozyme activity, even between individual members within one species (Rijkers, 1982). In this study, feeding of fish with 35 mg/kg vitamin E and 2.5 mg/kg folic acid showed better FWG, FCR, SGR and survival rate. Therefore, the supplemental levels of 35 mg/kg vitamin E and 2.5 mg/kg dietary folic acid are acceptable for use in rainbow trout diets, under farm conditions.

Table 4: Differential count of Lymphocyte of rainbow trout fingerlings fed 8 weeks with diets containing different levels of vitamin E and Folic Acid.

Treatments	Lymphocyte (%)	Neutrophil (%)
Control	99 ± 1	1 ± 1
T_1	100 ± 0.00	0.59 ± 0.15
T_2	99.3 ± 1.15	0.66 ± 1.1
T_3	99.3 ± 0.57	0.66 ± 0.57
T_4	100 ± 0.00	0.63 ± 0.05
T_5	98.6 ± 1.15	2.3 ± 0.53
T_6	99.6 ± 0.57	1.3 ± 1.1
T_7	98.3 ± 1.5	0.63 ± 0.53
T_8	98.6 ± 2.3	1.6 ± 0.56
T_9	99.6 ± 0.58	0.33 ± 0.58

Within a column, means with different superscripts are significantly different ($p< 0.05$).

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