The effects of different dietary vitamin C and iron levels on the growth, hematological and immunological parameters of rainbow trout *Oncorhynchus mykiss* fingerlings

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Abstract

A 60-days growth trial was conducted in a flow-through culture system to examine the effects of different dietary vitamin C and iron levels on the growth, hematological and immunological parameters of rainbow trout (*Oncorhynchus mykiss*) fingerlings (with average initial weight of 5.12 ± 0.02 g). Three levels of vitamin C (150, 200 and 250 mg kg^{-1} diet) and iron (5, 7 and 9 mg kg^{-1} diet) (as ferrous sulfate) and their combination were used to prepare diets used in nine experimental treatments with three replicates. The results revealed that the supplementation of basal diet with vitamin C and iron significantly affected the final body weight (FBW), total length (TL), feed conversion ratio (FCR) and specific growth rate (SGR). Among the haematological parameters haematocrit and mean corpuscular volume (MCV) were significantly influenced by vitamin C and iron. Results also showed that lysozyme and IgM concentrations significantly changed among dietary treatments. Based on the results of this study, it can be conclude that addition of vitamin C and iron to the basal diet of cultured rainbow trout will improve the growth rate and well-being of this fish.

Keywords: Growth performance, Hematological and immunological parameters, Vitamin C, Iron, Rainbow trout

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Introduction

The aquaculture industry is growing rapidly worldwide, but continues to contend with some issues such as disease, product quality, feed contamination and environmental impacts. Fish nutrition and its impact on animal welfare is an important aquaculture issue. Poor understanding of the fish dietary requirements in formulation and development of formulated artificial diets for intensive aquaculture have resulted in mortality rates (Halver, 2002). Vitamins are important essential nutrients for aquatic species. Several morphological and functional abnormalities have been reported in various fish species deprived of vitamins. In a research Lall and Ollivier (1993) concluded that in rainbow trout the disease resistance and hormonal antibody production was directly and positively related to the levels of vitamin C. It has been proven that dietary levels of vitamins C enhance the antibody production and immune memory in juvenile of milk fish (Azad *et al.,* 2007). Additionally, some previous research findings indicate that dietary supplementation with immunomodulatory vitamins such as vitamins Can improve the immune response and disease resistance of a variety of cultured fish species (Durve and Lovell, 1982; Verhlac *et al.,* 1998), while some other studies have failed to show positive responses with over fortification of such vitamins (Hardie *et al.,* 1990; Li *et al.,* 1998). So, it is clear that the biological role played by vitamins C is very vital for the sustained growth and health of many living organisms as well as fish. Nutrients do not function as independent units and have some interactions with other nutrients in terms of function and metabolism. Thus the requirement level of a particular nutrient may be affected by the level of another nutrient in either the diet or metabolically in the animal. There is some evidence that iron may be affecting the metabolism of ascorbic acid in the trout (Desjardins, 1985). However, it should be noted that this apparent interaction between iron and ascorbic acid metabolism most be due to the effects of iron supplementation on both the diet rancidity and on the stability of dietary ascorbic acid. However, there is still a paucity of information on the interaction of these two nutrients in fish. The objective of the present study was to assess the effects of different levels of dietary vitamin C and iron on the growth, hematological and immunological parameters of rainbow trout fingerlings.

Materials and methods

Experimental diets

The basal diet formula applied in this study is given in Table 1. Supplemental levels of vitamin C (L-ascorbyl-2-polyphosphate (LAPP) (STAY-C-35, 350 g kg^{-1} ascorbic acid equivalent, DSM, Netherland) at 150, 200 and 250 mg kg^{-1} and iron (ferrous sulfate, $FeSO_4H_2O$) at 5, 7 and 9 mg kg^{-1} were added to the basal diet to prepare nine experimental diets as follow: T_1; 5 mg Fe, T_2: 7 mg Fe, T_3: 9 mg Fe , T_4:150

mg vitamin C, T_5: 200 mg vitamin C, T_6: 250 mg vitamin C, T_7: 5 mg Fe + 150 vitamin C, T_8: 7 mg Fe + 200 mg vitamin C and T_9: 9 mg Fe + 250 mg vitamin C equivalent kg^{-1} diet. Also, a treatment without addition of vitamin C and iron was used as control. In preparing the diet, dry ingredients were first ground to small particle sizes in a mill. Ingredients were thoroughly mixed, and then fish oil and water were added to obtain a 25% moisture level. Following that, the diets were cold-pelleted into 3-mm diameter size using a pasta maker and then were dried in cool drier for 24 h. After drying, the diets were broken up and sieved into appropriate pellet sizes, and were stored in a cool place until used, and then from each diet one sample was taken for chemical analysis.

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

Ingredients	**g kg^{-1} dry weight**
Fish meal	620
Meat powder	60
Wheat flour	100
Soybean cake	50
Fish oil	40
Soybean oil	40
Lecithin	30
Vitamin mixture (Vitamin E free)[a]	15
Mineral mixture [b]	10
Salt	2
Proximate composition	%
Crude protein	49.18
Crude lipid	14.12
Moisture	14.25
Ash	20.70
Crude Energy (kcal kg^{-1})	3012

[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; MgSO4.7H2O, 7500; NaH2PO4.2H2O, 12 500; KH2PO4, 15 500; Ca(H2PO4)2ىH2O, 10 000; (CH2CHCOO)2Ca.5H2O, 1650; ZnSO4.7H2O, 176.5; MnSO4.4H2O, 81; CuSO4.5H2O, 16.5; CoCl2.6H2O, 0.53; KI, 1.59; starch, 147.5.

Experimental fish and feeding trial

The experiment was designed in nine treatments with three replicates for 8 weeks. A total of 810 Rainbow trout fingerlings (average weight: 5.12 ± 0.02 g; mean ± SD) were stocked into 27 fiberglass tanks (200L) at the Rainbow trout farm in Sari city (Mazandran, Iran). Fish were fed four times a day at 2% of body weight with adjustments made in quantity of feed supplied every week. Total fish weight in each tank was measured every 2 weeks for more accurate feeding rate adjustment. A diurnal 12-h l light: dark cycle was provided by fluorescent light, and dissolved oxygen, 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 hours and then weight end and total length (TL), weight gain (WG), final weight (FW), feed conversion ratio (FCR), specific growth rate (SGR), and condition factor (CF) and survival rate 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 weht (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 day.

Hematological parameters analysis

Fishes were anaesthetized in 100 ppm clove powder solution, 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 hematocrit (Ht), number of red blood cell (RBC) and total leukocyte count (WBC). Red blood cell (RBC, $x10^6$) and total leukocyte counts (WBC) were determined with a Neubauer using Rees diluting solution. To obtain the differential counts of leukocytes, that is, the number of lymphocyte, neutrophil, eosinophil and monocytes, we followed the following procedure. The prepared blood smears were first air dried, fixed in 96% ethanol for 30 minutes, stained by Giemsa staining solution for 30 minutes, and were examined under light microscope (Klontz, 1994). Hemoglobin concentration (Hb) was determined with Drabkin's reagent and read at absorbance at 540 nm (Jain, 1993). According to procedure of Rehulka (2000), haematocrit (Ht) was measured in microhaematocrit heparinized capillaries, using a microhematocrit centrifuge (13,000 rpm for 3 min). Mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) were obtained according to the method described by Haney *et al.* (1992).

Biochemical analysis

Blood was centrifuged at 3000 rpm for 15 minutes in cooling centrifuge for separation of plasma which was stored at -18ºC 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 hens egg white lysozyme (Sigma) in sterile sodium phosphate buffer. IgM content was determined following the method of Puangkaew *et al.,* 2004.

Data analysis

All data were subjected to a one-way analysis of variance (ANOVA) after confirmation of normality and homogeneity of variance. Significance of the differences between means was tested using Duncan's multiple range test ($p<0.05$). All assays were carried out in triplicates and data are shown as mean ± SD for each dietary group.

Results

The addition of different dietary vitamin C and iron levels to the basal diets significantly affected the FBW, TL, FCR and SGR; as these values were higher in fish fed diet containing 5 mg Fe + 150 vitamin C (T_7) than those fish fed the other treatment (Table 2). Red blood cells (RBC), White blood cell (WBC), hemoglobin (Hb), lymphocyte, monocyte, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were not affected by the supplemental vitamin C and iron levels (Tables 3 and 4). However, hematocrit (Hct) and mean corpuscular volume (MCV) were significantly influenced by different dietary vitamin C and iron levels. At the end of the experiment, significant differences were also observed in IgM (Fig. 1) and lysozyme (Fig. 2) in rainbow trout fed with diets containing different levels of vitamin C and iron.

Discussion

The results of the present study showed significant differences on FBW, TL, FCR and SGR among treatments with different dietary vitamin C and iron levels. Similarly, studies with common carp, (Gouillou-Coustans *et al.,* 1998) hybrid tilapia, *Oreochromis niloticus* (Shiau and Hsu, 2002), Japanese seabass, *Lateolabrax japonicus* (Ai *et al.,* 2004), juvenile grouper, *Epinephelus malabaricus* (Lin *et al.,* 2005) and juvenile cobia, *Rachycentron canadum* (Zhou *et al.,* 2012) indicate positive effect of vitamin C on the growth. Our results indicated that dietary vitamin C could improve the growth performance of juvenile rainbow trout. However, Ai *et al.,* (2006) reported that dietary vitamin C levels didn't influence the growth performance of the juveniles of large yellow croaker.

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 the experimental diets for 8 weeks.

Treatments	Initial weight (g)	Final weight (g)	WG (%)	TL (cm)	FCR	SGR (% day^{-1})	Condition factor	Survival rate (%)
Control	5.12 ± 0.02	24.6 ± 5.3 ^{c}	27.6 ± 0.71	13.5 ± 1.08	1.5 ± 0.04 ^{ab}	2.06 ± 0.25 ^{de}	1.04 ± 0.31	100
T_1	5.13 ± 0.02	25.8 ± 7.1 ^{d}	28.7 ± 1.1	14.1 ± 1.17	1.4 ± 0.03 ^{bc}	2.12 ± 0.33 ^{d}	1.04 ± 0.05	100
T_2	5.14 ± 0.01	27.1 ± 3.5 ^{e}	29.7 ± 1.3	14.2 ± 1.10	1.3 ± 0.01 ^{bcd}	2.18 ± 0.15 ^{cd}	0.96 ± 0.02	100
T_3	5.12 ± 0.06	28.2 ± 7.5 ^{f}	30.7 ± 1.8	14.9 ± 0.77	1.2 ± 0.02 ^{cd}	2.23 ± 0.32 ^{e}	1.04 ± 0.09	100
T_4	5.15 ± 0.02	21.6 ± 4.9 ^{a}	25.3 ± 1.4	13.6 ± 0.17	1.6 ± 0.04 ^{a}	1.91 ± 0.25 ^{f}	1.03 ± 0.11	100
T_5	5.10 ± 0.05	23 ± 9.9 b ^{g}	26.3 ± 1.6	13.4 ± 1.53	1.4 ± 0.04 ^{bc}	1.99 ± 0.49 ^{e}	1.06 ± 0.16	100
T_6	5.11 ± 0.04	31± 7.9 b ^{ef}	33 ± 1.2	15.3 ± 0.60	1.1 ± 0.02 ^{e}	2.36 ± 0.32 ^{a}	1.12 ± 0.06	100
T_7	5.12 ± 0.03	29.4 ± 4.1 ^{d}	31.5 ± 1.09	14.6 ± 0.78	1.2 ± 0.01 ^{cd}	2.28 ±0.17 ^{b}	1.13 ± 0.02	100
T_8	5.13 ± 0.02	27.6 ± 1.9 ^{d}	30.1 ± 0.61	14.5 ± 0.96	1.3 ± 0.02 ^{bcd}	2.21± 0.08 ^{c}	0.93 ± 0.03	100
T_9	5.13 ± 0.01	25.8 ± 3.3 ^{c}	28.7 ± 1.1	13.8 ± 0.96	1.3 ± 0.01 ^{bcd}	2.12 ± 0.15 ^{d}	1.04 ± 0.07	100

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

Table 3: Hematological parameters of rainbow rout fingerling fed 8 weeks with diets containing different levels of vitamin C and iron.

Treatments	RBC (×10^{6})	WBC (×10^{3})	Hematocrit (%)	Hemoglobin (gr/dl)	MCV (fl)	MCH (pg)	MCHC (gr/dl)
Control	101 ± 21	67.6 ± 15	36 ± 2.6 ^{e}	6.3 ± 0.49	299 ± 21.1 ^{bcd}	52.6 ± 6.3	17.5 ± 1.6
T_1	97 ± 16.2	69.6 ± 8.9	33.3 ± 2.8 ^{cde}	6.5 ± 0.42	289 ± 38.2 ^{bcd}	56.4 ± 7.1	19.5 ± 0.2
T_2	102 ± 21.5	69.3 ± 14	31.6 ± 1.1 ^{cd}	5.7 ± 1.2	303.8 ± 18.7 ^{d}	59.1 ± 6.2	17.9 ± 2.2
T_3	101 ± 10.3	64 ± 15	28.3 ± 1.5 ^{ab}	5.9 ± 0.13	316 ± 49.5 ^{cd}	59.5 ± 10.3	18.8 ± 0.36
T_4	103 ± 12.7	64 ± 16.2	32 ± 1 ^{cd}	5.9 ± 0.81	244 ± 11.5 ^{ab}	51.1 ± 6.7	20.9 ± 2.01
T_5	101 ± 21.7	64.6 ± 15.5	30.6 ± 1.1 ^{bcd}	6.1 ± 0.11	300 ± 25.7 ^{bcd}	56.1 ± 8.7	18.6 ± 1.8
T_6	100 ± 13.9	61 ± 12.2	29 ± 4 ^{abc}	5.8 ± 0.49	273 ± 10.2 ^{abc}	54.2 ± 0.09	19.9 ± 0.76
T_7	102 ± 16	62.6 ± 9.2	28 ± 1 ^{a}	5.7 ± 0.04	298 ± 51.6 ^{bcd}	61.1 ± 15.8	20.2 ± 2.02
T_8	103 ± 10.3	66 ± 7.5	33.3 ± 2.3 ^{cde}	6.2 ± 1.4	232 ± 20.2 ^{a}	47.9 ± 2.4	20.6 ± 0.75
T_9	102 ± 17.3	64 ± 13.7	36 ± 2.8 ^{e}	6.3 ± 0.49	254 ± 14.1 ^{ab}	46.9 ± 9.1	18.6 ± 4.7

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

Table 4: Differential count of leukocyteof of rainbow rout fingerling fed 8 weeks with diets containing different levels of vitamin C and iron.

Treatments	Monocyte (%)	Neutrophil (%)
Control	99 ± 1	1 ± 1
T_1	99.5 ± 0.58	0.67 ± 0.57
T_2	98 ± 1.03	1 ± 1
T_3	98.6 ± 1.5	1.3 ± 0.53
T_4	99.6 ± 0.58	0.33 ± 0.05
T_5	98.3 ± 2.08	2 ± 0.73
T_6	99.3 ± 1.1	0.67 ± 0.16
T_7	98.3 ± 1.5	1.67 ± 0.53
T_8	99 ± 1	1 ± 1
T_9	99.6 ± 0.58	0.33 ± 0.58

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

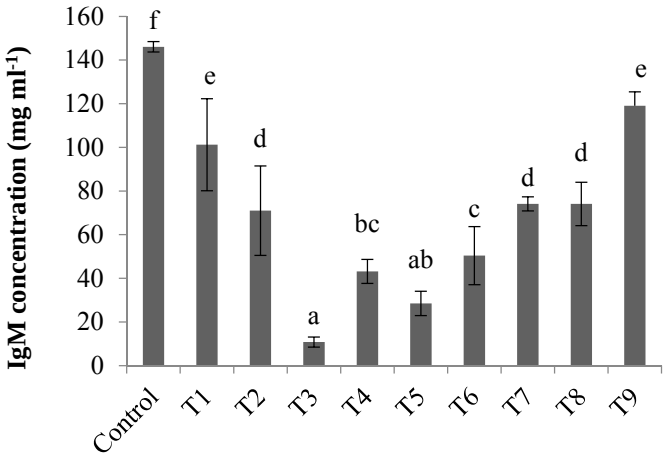

Figure 1: Total IgM concentration of concentration of rainbow rout fingerling fed 8 weeks with diets containing different levels of vitamin C and iron.

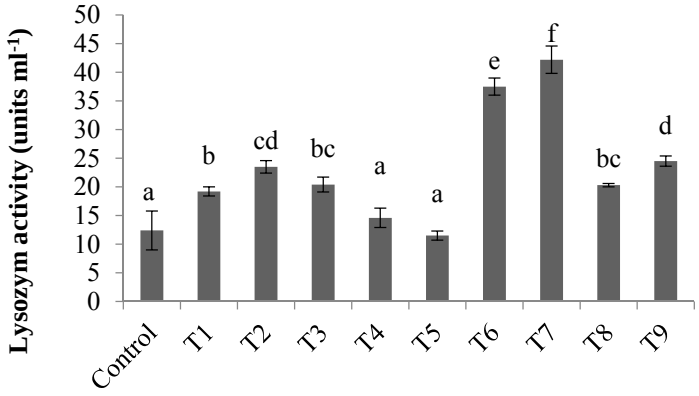

Figure 2: Lysozyme activity of rainbow rout fingerling fed 8 weeks with diets containing different levels of vitamin C and iron.

Studies with common carp (Sakamoto and Yone, 1978a), red sea bream (Sakamoto and Yone, 1978b), Atlantic salmon (Andersen *et al.,* 1996), channel catfish (Barros *et al.,* 2002) and juvenile gibel carp, *Carassius auratus gibelio* (Pan *et al.,* 2009) did not indicate an adverse effect of iron deficiency on the growth performance. However, Shiau and Su, (2002) reported that weight gain of Nile tilapia increased with dietary iron while significantly decreasing at iron levels. These discrepancies might be due to differences in individual size, development stage, and cultivation environment. There were significant differences in Hct and MCV values among our treatments. Similar results were observed in pirarucu (*Arapaima gas*) and cobia juvenile's *R. canadum* (Zhou *et al.,* 2012) fed with diet not supplemented with vitamin C. In agreement with our results Pan *et al.,* (2009) also have reported that addition of iron to the basal diet significantly affected the Hct in juvenile gibel carp, *C. auratus gibelio*. Vitamin C is a powerful antioxidant protecting against oxidative damage to various tissues of fish including red blood cells (Sahoo and Mukherjee, 2003). Lower red blood cells were regarded as the sun of anemia which has been reported in most studies with fish where vitamin C deficiency was observed (NRC, 2011). Moreover, Adham *et al.* (2000) demonstrated that feeds with insufficient vitamin C cause anemia, characterized by a decrease in the hemoglobin, reduction in the number of erythrocytes and hematocrit. Many researchers have shown that ascorbic acid is involved in the metabolism of iron in fish and a deficiency of ascorbic acid has been observed to cause a reduction in serum iron levels and a redistribution of tissue iron stores in

rainbow trout (Hilton *et al.,* 1989) and a reduction in both hemoglobin and hematocrit levels in catfish, trout and snakehead fish (Lim and Lovell, 1978; Agrawal and Mahajan, 1980). There are also some as that may indicate the effect of iron on ascorbic acid metabolism in trout. Despite the interaction of ascorbic acid and iron metabolism in fish, it is interesting to note that increasing the levels of ascorbic acid do not appear to affect the absorption of dietary iron in fish (Lanno *et al.,* 1985). The innate immune system of fish is regarded to be the first line of defense against pathogens and is more important for fish as compared with mammals. Lysozyme is released by leukocytes and plays a crucial role in antimicroorganism activity. In the present study, fish fed with different dietary vitamin C and iron levels had lower lysozyme activity than fish fed with the basal diets. Previous studies indicated that the lysozyme activity have been found to be positively correlated with supplementation of ascorbic acid (Roberts *et al.,* 1995; Ortuno *et al.,* 1999; Anbarasu and Chandran, 2001; Ai *et al.,* 2004; Ai *et al.,* 2006). In the present study, rainbow trout fed the basal diet had higher concentration than fish fed the other diets. Contrary to our results, Zhou *et al.,* (2012) showed that cobia, *R. canadum* fed with diet not supplemented with vitamin C had lower concentration than fish fed the other diets. In conclusion, more studies using basal diets containing different vitamins and minerals should be carried out to examine the effect of dietary vitamins and minerals on juvenile rainbow trout growth performance and haematological parameters.

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