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Effects of Different Levels of Dietary Vitamins C and E on Some of Hematological and Biochemical Parameters of Sterlet (*Acipenser ruthenus*)

[1]M. Tatina, [2]M. Bahmani, [3]M. Soltani, [4]B. Abtahi and [5]M. Gharibkhani
[1]Department of Fisheries Science, Faculty of Agriculture and Natural Resources, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2]International Sturgeon Research Institute, Rasht, Iran
[3]Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Iran
[4]Department of Marine Biology, Faculty of Biological Science, Shahid Beheshti University, G.C., Tehran, Iran
[5]Young Researchers Club, Islamic Azad University, Astara Branch, Astara, Iran

Abstract: This study was conducted in order to characterize the different levels of dietary vitamin C and vitamin E on some of hematological and biochemical parameters of sterlet. For this purpose 270 sterlet (*Acipenser ruthenus*) were divided into 18 groups. Three levels of vitamin E (0, 100 and 400 mg kg^{-1} diet) and vitamin C (0, 100 and 400 mg kg^{-1} diet) and their combination were used to prepare nine experimental diets. Each of nine experimental diets was fed to fish in 2 tanks (2 replicates). The fish were fed 3% of their wet b.wt. per day for a 100 days period. Blood samples were obtained from three fish of each tank at the end of experiment. The results reveal that Fish fed diets containing 100 mg kg^{-1} vitamin E and 400 mg kg^{-1} vitamin C (diet 7) had the highest WBC ($p>0.05$). Also, significantly higher RBC was observed in diets 3, 4, 5, 6 and 9 than those of the other diets in which different levels of each vitamin without any regulation exists. The hematocrit percentage did not differ significantly in fish fed the different diets ($p<0.05$). Also, there was no significant difference in the mean amount of total protein, cortisol, glucose and triglyceride between the fish fed with the different diets designed for this experiment ($p>0.05$). On the other hand, fishes fed diets without vitamin C but different levels of vitamin E (diets 3 and 6) had significantly higher amounts of cholesterol compared with fish fed with other diets.

Key words: Sterlet, vitamin C, vitamin E, hematological, biochemical

INTRODUCTION

Nutritional requirements of an animal are a fundamental aspect that depends on species, habitat and live cycle stage (Sargent *et al*., 1989). Vitamins are important essential nutrients for most animal species. Vitamin deficiencies in fish under aquaculture are known to produce biochemical dysfunction leading to tissue and cellular level clinical manifestations. Several morphological and functional abnormalities have been reported in various fish species

Corresponding Author: M. Tatina, Department of Fisheries Science, Faculty of Agriculture and Natural Resources, Science and Research Branch, Islamic Azad University, Tehran, Iran

deprived of vitamins. Dietary vitamins were reported to have antibody enhancement effects in salmon (Navarre and Halver, 1989; Wagbo *et al*., 1993). Properties of disease resistance in fish fed vitamin C and E have been reported by several researchers (Hardie *et al*., 1983, 1990; Navarre and Halver, 1989; Satyabudhy *et al*., 1989; wagbo *et al*., 1993). Disease resistance and humoral antibody production in rainbow trout was directly and positively related to the levels of vitamin C in the trout diet (Lall and Olivier, 1993). It was also shown that supra dietary levels of vitamins C and E may enhance antibody production and immune memory in juvenile milk fish to formalin-killed *Vibrio vulnificus* (Azad *et al*., 2007). So, it is clear that the biological role played by vitamins C and E as two important vitamins is very vital for the sustained growth and health of many living organisms as well as fish. These two vitamins 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 (Burton *et al*., 1983; Frei *et al*., 1990). This is why both vitamins have been known for protective actions against free radicals. The beneficial role of these two vitamins have been extensively reported in fish nutrition, reproduction (Wilson *et al*., 1984; Dabrowski and Ciereszko, 2001; Halver, 2002) growth and related indices (Martinez, 1990; Roem *et al*., 1990; Thorarinsson *et al*., 1994; Chien *et al*., 1999; Lim *et al*., 2000; Xie *et al*., 2006; Sau *et al*., 2004; Belo *et al*., 2005; Tocher *et al*., 2002). In addition, vitamins E and C are credited with modulating the stress response in fish (Merchie *et al*., 1997; Montero *et al*., 1998, 2001; Kolkovski *et al*., 2000; Ortuno *et al*., 2003).

Interactions between the two vitamins have been proven in many *in vivo* studies since Packer *et al*. (1979) demonstrated that vitamin C spares vitamin E by regenerating it from tocopheroxyl radicals. Further studies attempted to verify the interaction between vitamin C and E *in vivo* in several animal species (Igarashi *et al*., 1991; Liu and Lee, 1998). In Atlantic salmon (Hamre *et al*., 1997) and rainbow trout (Furones *et al*., 1992), vitamin C intake above the requirement for growth did not influence tissue vitamin E levels, irrespective of their vitamin E status. However, dietary vitamin C prevented the appearance of vitamin-E-deficiency signs in Atlantic salmon in a dose-dependent manner (Hamre *et al*., 1997). Hepatic vitamin E concentrations of normal lake sturgeon and vitamin-E-deficient yellow perch increased in fish fed high dietary concentrations of vitamin C (Moreau *et al*., 1999; Lee and Dabrowski, 2003). However, hepatic vitamin E concentration decreased with increasing dietary vitamin C in hybrid striped bass (Sealey and Gatlin, 2002). Vitamin C deficiency also developed earlier in Atlantic salmon fed a diet high in vitamin E due to the accumulation of the vitamin E radical (tocopheroxyl) which is otherwise reduced by vitamin C (Hamre *et al*., 1997).

Some studies on this association have demonstrated that there are two interaction mechanisms between vitamins C and E: a synergistic simultaneous protection effect of the lipid and aqueous phases against oxidation and the action of vitamin C on vitamin E regeneration in the tissues. Data on growth, mortality, hematology and lipid oxidation in the liver have demonstrated that vitamin C protected fish against vitamin E deficiency (Hamre *et al*., 1997; Shiau and Hsu, 2002). Interaction between these two vitamins is also known to influence the beneficial effects they induce in cultured fish. Vitamin C/E sparing action in channel catfish was studied to explain the variability observed in its sensitivity to Vitamin E deficiency (Lovell *et al*., 1984). So, due to their potential for interaction, dietary requirements for vitamins C and E are often considered together.

Sturgeons are a very ancient fish group, existing since the Late Cretaceous with a wide distribution in the Northern Hemisphere (Grande and Bemis, 1991). Sturgeon biology is

interesting because of important conservation and economic issues involving these fishes. Sturgeons are the source of two high-value products: boneless and very tasteful meat and black caviar. Fish farms producing sturgeon meat and caviar as well as reproducing sturgeons under controlled conditions have emerged (Wade and Fadel, 1997; Chebanov and Billard, 2001). *Acipenser ruthenus* is the smallest species of sturgeons and relatively short lived. The sterlet is a potomodromous resident of large rivers flowing into the Caspian, Black, Barents and Kara seas (Peterson *et al*., 2006).

Despite the economic importance of sterlet and its culture there is not enough information on the vitamin requirements of this species. So this study was conducted in order to characterize the different levels of dietary vitamin C (0, 100 and 400 mg kg^{-1} diet) and vitamin E (0, 100 and 400 mg kg^{-1} diet) on some of hematological and biochemical parameters of sterlet.

MATERIALS AND METHODS

Fish Preparation

The experiments were performed in the Breeding Center of International Sturgeon Research Institute (Rasht, Iran) in January up to July 2008. Two hundred and seventy sterlet (*Acipenser ruthenus*) weighing 364.2±0.23 g were sorted, divided into 18 groups (15 fish per group) and tagged. Each fish group were stocked in 2 tonne fiberglass tanks supplied with filtered Sefidroud river water (DO: 10.4 mg L^{-1}; pH: 7.2 and T: 7.1±1°C) for acclimation. During this period (2 weeks) fish were fed three times a day with basal diet at a rate of 3% b.wt. day^{-1}.

Experimental Diets

Three levels of vitamin E (0, 100 and 400 mg kg^{-1} diet) and three levels of vitamin C (0, 100 and 400 mg kg^{-1} diet) and their combination were used to prepare nine experimental diets out of which one diet contains only basal diet and the other 8 diets consists of basal diet and different levels of vitamin C and E. The proximate composition (AOAC, 2000) of the basal diet was: moisture 15%, crude protein 46%, crude lipid 17.1%, crude ash 8.7% and crude fiber 2.2%. Table 1 shows the composition of nine experimental diets. The diets were

Table 1: Composition of the experimental diets

	Diet								
Diets ingredients	1	2	3	4	5	6	7	8	9
Fish meal (%)	54.0	54.0	54.0	54.0	54.0	54.0	54.0	54.0	54.0
Wheat meal (%)	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0
Powdered milk (%)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Soybean meal (%)	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Wheat gluten (%)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Fish oil (%)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
yeast (%)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Mineral mixture[a] (%)	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
Vitamin mixture[b] (%)	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7
Vitamin E[c] (mg kg^{-1})	0.0	0.0	100.0	100.0	0.0	400.0	100.0	400.0	400.0
Vitamin C[d] (mg kg^{-1})	0.0	100.0	0.0	100.0	400.0	0.0	400.0	100.0	400.0

[a]Mineral mixture (mg g^{-1} mixture): Calcium lactate, 327; K_2PO_4, 239.8; $CaHPO_4$. $2H_2O$, 135.8; $MgSO_4$. $7H_2O$, 132; Na_2HPO_4. $2H_2O$, 87.2; NaCl, 43.5; Ferric citrate, 29.7; $ZnSO_4$. $7H_2O$, 3; $COCl_2$. $6H_2O$, 1; $MnSO_4$. H_2O, 0.8, KI, 0.15; $AlCl_3.6H_2O$, 0.15; $CuCl_2$, 0.1. [b]Vitamin mixture (mg g^{-1} mixture): Thiamin hydrochloride, 2.5; Riboflavin, 10; Calcium pantothenate, 25; Nicotinic acid, 37.5; Pyridoxine by hydrochloride, 2.5; Folic acid, 0.75; Inositol, 100; Chlorine chloride, 250; Menadione, 2; Retinol acetate, 1; Cholecalciferol, 0.0025; Biotin, 0.25; Vitamin B_{12}, 0.05. All ingredients were diluted with alpha-cellulose to 1 g. [c]Vitamin E: D-Alpha-tochopherol. [d]Vitamin C: L-Ascorbil-2-polyphosphate

prepared weekly in the laboratory in pellet form. Each of nine experimental diets was fed to fish in 2 tanks (2 replicates). The fish were fed 3% of their wet b.wt. day^{-1}. This amount was similar to feed consumption during the acclimation period. The feed was divided into three equal meals and fed at 08:00, 16:00 and 24:00 h. Fish were weighed once every two weeks and the daily ratio adjusted accordingly. The fish were fed the test diets for a 100 days period. During this period no mortality was occurred in the treatment tanks.

Hematological and Biochemical Assay

Blood samples were obtained from fish at the end of experiment. Fishes were fasted for 24 h before sampling. Three fish of each tank were randomly chosen and anesthetized with tricaine methanesulfonate (MS-222; Argent Chemical, Redmond, WA) at 125 mg L^{-1}. Five milliliters of blood samples were collected from the caudal vein with heparinized 27-gauge needles for determination of hematocrit (HCT), White Blood Cell (WBC) and Red Blood Cell (RBC) counts, total protein, triglyceride, cortisol, glucose and cholesterol. The HTC was determined by the microhematocrit method described by Rehulka (2000). The WBC and RBC were determined by diluting blood and enumeration in a hemacytometer. Remaining clotted blood was centrifuged at 3000x g for 15 min and plasma was removed and stored at -20°C for further analysis. Total serum protein was determined using refractometer (model SPR-NE, Japan). Total plasma cortisol level were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Neogen Corp., Lexinton, KY, USA), read at 650 nm (Basu *et al*., 2001). Also, Plasma glucose, cholesterol and triglyceride were determined enzymatically using spectrophotometer (model RA-1000, Technicon Corp., USA) and Pars Azmun kits (Iran).

Statistical Analysis

Data were analyzed by two-way analysis of variance (ANOVA) with 3 levels of vitamin C and 3 levels of vitamin E (3×3 factorial experiment) using the SAS/PC statistical software (SAS, 1993) and significance was set at $p<0.05$. This analysis allowed examination of the main effects of vitamin C and E as well as any interactions occurring between the two factors. When a significant main effect was found without the interaction effect, the differences between treatment means were determined by Duncan's multiple range tests. If a significant interaction was observed, the differences between simple effects were determined by Student's t-test.

RESULTS

Influence of Different Diets on Hematological Parameters

Mean final hematological parameters (WBC, RBC and HCT) are given in Table 2. Fish fed diets containing 100 mg kg^{-1} vitamin E and 400 mg kg^{-1} vitamin C (diet 7) had significantly the highest WBC, but fish fed diets containing 400 mg kg^{-1} vitamin E and 400 mg kg^{-1} vitamin C (diet 9) had the lowest WBC. There was no significant difference in the number of WBC between other 7 diets. On the other hand the significantly highest RBC was observed in diets 3, 4, 5, 6 and 9 in which different levels of each vitamin without any regulation exists. The percent of hematocrit did not significantly differ in fish fed different diets. This indicates that in these trial different levels of vitamin C and E had no effect on the HTC.

Influence of Different Diets on Biochemical Parameters

Mean final biochemical parameters (total protein, cortisol, glucose, cholesterol and triglyceride) are given in Table 3. The results showed that there was no significant difference

Table 2: The Mean±SD of hematological parameters in fish fed with different diets for 100 days

Treatments	WBC	RBC	HCT (%)
	(Cell μL^{-1})		
Diet 1	18250.0±3083.3ab	748333.0±71666.7b	29.1±0.1
Diet 2	13750.0±583.3ab	793333.0±10000.0b	28.5±1.5
Diet 3	15250.0±2750.0ab	1255000.0±11666.7a	31.1±0.1
Diet 4	18583.3±1750.0ab	1140000.0±70000.0a	28.0±0.3
Diet 5	14250.0±1750.0ab	1116667.0±56666.7a	29.8±0.8
Diet 6	14666.7±1333.3ab	1185000.0±108333.0a	29.1±0.5
Diet 7	19666.7±1666.6a	748333.0±71666.7b	32.0±2.6
Diet 8	14250.0±1750.0ab	793333.0±10000.0b	28.5±1.5
Diet 9	13000.0±166.6b	1180000.0±110000.0a	32.0±2.6

Values within the same column with different letter(s) are significantly different ($p>0.05$)

Table 3: The Mean±SD of biochemical parameters in fish fed with different diets for 100 days

Treatments	Total protein ($g\ dL^{-1}$)	Cortisol ($ng\ mL^{-1}$)	Glucose	Cholesterol ($mg\ dL^{-1}$)	Triglyceride
Diet 1	4.5±0.0	42.1±3.8	116.6±3.3	23.3±1.6b	274.3±55.6
Diet 2	4.8±0.5	44.0±8.0	210.3±126.3	27.9±5.0ab	538.3±253.6
Diet 3	4.9±0.2	34.5±0.5	215.0±80.3	46.3±10.3a	462.1±106.1
Diet 4	4.9±0.0	44.6±2.6	131.5±16.1	18.7±1.9b	347.6±65.0
Diet 5	4.5±0.0	38.5±0.8	133.6±20.6	36.3±9.6ab	303.3±57.6
Diet 6	4.2±0.3	37.5±2.5	111.5±13.5	45.3±3. a	304.0±30.6
Diet 7	4.9±0.0	44.6±2.3	131.3±15.6	44.6±2.3ab	347.6±65.0
Diet 8	4.5±0.0	39.6±0.6	133.3±20.6	39.6±0.6b	303.3±57.6
Diet 9	4.6±0.2	36.5±5.5	204.0±65.5	35.5±5.5b	399.6±41.0

Values within the same column with different letter(s) are significantly different ($p>0.05$)

in the mean amount of total protein, cortisol, glucose and triglyceride between the fish fed with the different diets designed for this experiment. On the other hand fish fed diets containing no vitamin C but different levels of vitamin E (diets 3 and 6) had significantly the highest amount of cholesterol compared with fish fed with other diets. This means that different levels of vitamin C used in our trial had no effect on the cholesterol amount. But diets containing excessive amounts of vitamin E (diet 8 and 9) and the basal diet without any vitamin supplement (diet 1) had the lowest significant amount of cholesterol.

DISCUSSION

Vitamin C is synthesized in animals from either D-glucose or D-galactose as part of the glucuronic acid pathway (Lehninger and Hassan, 1956). Branching from L-gulonic acid, the biosynthetic pathway of vitamin C comprises three consecutive steps: first, the enzymatic lactonization of L-gulonic acid catalyzed by L-gulonolactone hydrolase (Stubbs and Haufrect, 1968), second, the oxidation of L-gulonolactone catalyzed by L-gulonolactone oxidase (GLO) and third, the spontaneous isomerization of 2-keto-L-gulonolactone leading to vitamin C (Chatterjee *et al*., 1960). The general view is that animals lacking GLO are unable to synthesize vitamin C and thus depend upon a dietary source of the vitamin (Sato *et al*., 1976). Among the fishes analyzed to date, only those retaining numerous ancestral characters, such as lamprey, shark, ray, lungfish and sturgeon (Dykhuizen *et al*., 1980; Dabrowski, 1994; Touhata *et al*., 1995; Moreau *et al*., 1996; Moreau and Dabrowski, 1998; Maeland and Waagbo, 1998) have been shown to have GLO in the kidney, whereas teleost fish lack GLO activity (Dabrowski, 1990). Dabrowski (1994) showed that in a 6-month-old white sturgeon fed a diet devoid of vitamin C, tissue total vitamin C concentrations were not decreased suggesting that white sturgeon GLO produced adequate amounts of vitamin C to meet the fish needs at a stage of rapid growth while the dietary source was withheld. On the

other hand, some studies were carried out on tissue concentration of vitamin E in some species of sturgeon fed with different levels of this vitamin, but none of them could determine its accurate requirements (Webster and Lim, 2002).

The results of this study on hematological parameters revealed that neither dietary levels of vitamin C nor vitamin E influences HCT in sterlet. Similar results were obtained on HCT in juvenile golden shiner (Chen *et al*., 2004) fed with different levels of vitamin C and E , sturgeon hybrid (Jeney and Jeney, 2002) fed with different levels of vitamin C and Atlantic halibut (McCrea and Hall, 2007) fed with different levels of vitamin E.

The highest number of WBC was observed in fish fed with diet 7 containing 100 mg kg^{-1} vitamin E and 400 mg kg^{-1} vitamin C. In a similar study on the effect of different levels of vitamin E on immune response of grouper, Lin and Shiau (2005) showed that by increasing vitamin E in diet, the fish WBC count was increased accordingly, but the dietary vitamin E supplemented level was not determined accurately. Also, like persent study, Falahatkar (2005) in his study on the effect of different levels of diet vitamin C on some of hematological parameters of great sturgeon found that there were significant differences in WBC among the treatments.

The highest number of RBC was observed in different diets other than basal diet which shows that diets containing different levels of vitamin C and vitamin E have significant influence on RBC value. Similar results were obtained by Lim *et al.* (2000) for channel catfish fed with different vitamin C and iron concentrations, Falahatkar (2005) for great sturgeon fed with different vitamin C levels, Montero *et al.* (2001) for *S. aurata* fed with different vitamin C and E concentrations and Andrade *et al.* (2007) for pirarucu fed with different vitamin C and E concentrations.

The results of this study on biochemical parameters revealed that neither dietary levels of vitamin C nor vitamin E influences total protein, cortisol, glucose and triglyceride in sterlet. Andrade *et al.* (2007) reflected that high vitamins C and E concentrations could stimulate protein production in fish, suggesting an important role of both vitamins in the modulation of plasma proteins. But our study showed that plasma protein was not significantly affected by dietary vitamin C and E levels. Similar results were obtained by Sealy and Gatlin (2002) for hybrid striped bass, Garcia *et al.* (2007) for *Piaractus mesopotamicus* and Chen *et al.* (2004) for golden shiner all fed diets supplemented with different levels of vitamins C and E.

In fish, glucose concentration may vary greatly depending on the physiological status of the animal. According to Mommsen *et al.* (1999), plasma glucose levels can increase, decrease, or keep constant under high plasma cortisol. *Piaractus mesopotamicus* fed with diets containing 100 or 450 mg kg^{-1} vitamin E did not show a glycemic alteration compared to fish fed diets without this supplementation (Belo *et al*., 2005).Similarly the results of persent study reveals that different levels of vitamin C and E had not any significant influence on plasma glucose. On the Contrary Andrade *et al.* (2007), showed that the plasma glucose concentrations in pirarucu were elevated in 800 and 1200 mg vitamin E kg^{-1} treatments in relation to the control group and to those with vitamin C. Therefore, it is not possible to confirm whether hyperglycemia is an advantage for these animals, since there is still a lack of standardization on vitamin E supplementation, which metabolic implications are yet to be determined.

Generally, it is assumed that the nutritional state of a fish can affect the animal health and possibly the way they deal with stress. The stress response in fish is generally mediated by a neuroendocrine response, which includes the release of stress hormones such as cortisol and catecholamines into the circulatory system (Barton and Iwama, 1991; Barton, 2002). These and possibly other hormones, elicit several compensatory physiological

responses that help the fish to deal with the stressor. Glucose is one of the most important energy substrates used by fish to cope with physiological stress and therefore plasma glucose levels have been used as an indicator of the stress response. It was demonstrated that cortisol and glucose could increase in teleost exposed to stress (Mommsen *et al*., 1999). So one of the reasons that our fish fed different levels of dietary vitamin C and E did not show any significant differences in cortisol and glucose values, may be because stress was avoided during the experiment in all of the treatments.

The results of present study showed that different levels of vitamin C used in our trial had no effect on the cholesterol amount. But diets containing excessive amounts of vitamin E (diet 8 and 9) and the basal diet without any vitamin supplement (diet 1) had the lowest significant amount of cholesterol which reveals that only a optimum amount of vitamin E can increase cholesterol and excessive amounts can lead to its decline. It had been demonstrated that high levels of dietary vitamin E may decrease the amount of triglyceride and cholesterol in humans (Haglund *et al*., 1991). But an organized study on the effect of dietary levels of vitamin C and vitamin E on the triglyceride and cholesterol values of fish have not been carried out yet.

It had been documented that different factors are effective on the hematological and biochemical parameters of fishes, from which the species, environmental condition, age, maturation and nutrition are very important (Ross and Ross, 1999). The studies on determining the dietary vitamin requirements of sturgeon are scarce. The data generated in this study showed that dietary levels of vitamin C and vitamin E may have influence on some of hematological and biochemical parameters of sterlet. The different results obtained in this study from others reveals that the mechanism of vitamins C and E interaction in sterlet as a sturgeon may differ from bony fishes due to the ability of these species to synthesize vitamin C. On the other hand most of similar studies on fishes were carried out on fingerlings and in some cases juveniles but the fishes used in this study were big and there is no precise information on the vitamin requirements of these size of sturgeon fishes yet. So, in order to have better knowledge about the nutritional requirements of sturgeon as well as sterlet further studies are recommended.

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