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Effect of different levels of iron sulfate on some haematological parameters of ship sturgeon, *Acipenser nudiventris*

Hosseini S.H.[1]; Kamali A.[1*]; Yazadani M.A.[2]; Khara H.[3]

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Abstract

The present study was conducted to examine different dietary levels of iron ($FeSO_4$) on some haematological parameters of ship sturgeon, *Acipenser nudiventris* including red blood cells (RBC), white blood cells (WBCs), differential WBCs, hematocrit (Hct), Hemoglobin (Hb), the mean corpuscular hemoglobin (MCH) and the mean corpuscular hemoglobin concentration (MCHC), the mean corpuscular volume (MCV), immunoglobulin (IgM), ferritin, transferrin and plasma Fe^{2+}. Four experimental treatments and one control group with three replicates were considered for the experiment. The experimental treatments were fish fed experimental diets containing different levels of $FeSO_4$ i.e. 100, 200, 250, and 300 mg kg.diet^{-1}. After 60 days, there were significant differences between experimental groups in terms of WBCs, lymphocytes, neutrophils, monocytes, eosinophils, MCH and MCHC, MCV, total plasma protein, IgM, ferritin, transferrin and plasma Fe^{2+} (p<0.05). In the treatment fed 100 mg.iron kg.diet^{-1}, more levels of IgM and total plasma protein were observed compared to other experimental groups (p<0.05). The highest values of neutrophils, monocytes and MCHC were found in control fish whereas MCV, MCH and eosinophils values were higher in the treatment fed 200 mg.iron kg.diet^{-1} (p<0.05). Also, more WBCs were observed in 250 mg.iron kg.diet^{-1} treatment than in other groups (p<0.05). The values of lymphocytes, RBC, Hct, Hb, ferritin, transferrin and plasma Fe^{2+} were higher in fish fed 300 mg.iron kg.diet^{-1} compared to other experimental groups (p<0.05). In conclusion, our results showed that the supplementation of ship sturgeon diets with high levels of iron enhances the RBC, Hct, Hb, ferritin, transferrin and plasma iron.

Keywords: Iron sulfate ($FeSO_4$), Haematological parameters, Ship sturgeon, *Acipenser nudiventris*

1-Department of Fisheries, Science and Research Branch, Islamic Azad University, Tehran, Iran, P.O.Box:14515/775

2-Department of Aquaculture, International Sturgeon Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Rasht, Iran, P.O. Box: 41635-3464

3-Department of Fisheries, Lahijan Branch, Islamic Azad University, Lahijan, Iran, P.O. Box: 1616

*Corresponding author's Email: Kamali.abolghasem@gmail.com

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Introduction

Iron as one of the essential minerals plays an important role in many physiological and functional processes of living organisms including oxygen transport, cellular respiration and lipid oxidation reactions (Lee *et al.*, 1981). However, little information is available on role of iron in biological process of fish (Lall, 2002). In fish, iron deficiency has led to some pathological impacts including haematological suppressions such as microcytic anemia or low haemoglobin levels (Kawatsu, 1972; Ikeda *et al.*, 1973; Sakamoto and Yone, 1976; Nose and Arai, 1979; Gatlin and Wilson, 1986; Davis and Gatlin, 1991; Andersen *et al.*, 1996; Watanabe *et al.*, 1997), reduced haematocrit, depletion of hepatic iron (Bjørnevik and Maage, 1993; Andersen *et al*., 1996) and at higher levels reduced growth and poor feed conversion (Tacon, 1992). Also, many studies have reported the harmful effects of the excessive amounts of dietary iron in fish (Salte *et al.*, 1994; Andersen *et al.*, 1997) such as increases in gut bacterial load (Fouz *et al.*, 1994) and lipid peroxidation in the liver (Baker *et al.*, 1997). Thus, determining the optimum dietary iron requirement is essential for the performance of any farmed fish of interest. Fish acquire iron predominantly from the diet, and with negligible iron uptake at the gills compared with the gut (Andersen *et al*., 1997; Bury and Grosell, 2003). The requirements of iron have been determined for several fish species. Generally, teleost fish have a dietary iron requirement of 30–200 mg kg.diet^{-1} (Davis and Gatlin, 1991; Watanabe *et al.*, 1997). In detail, the minimum dietary iron requirement has been reported to be between 60 and 100 mg kg^{-1} in Atlantic salmon, *Salmo salar* (Andersen *et al.*, 1996), 30 mg kg^{-1} in channel catfish, *Ictalurus punctatus* (Gatlin and Wilson 1986), 170 mg kg^{-1} in Japanese eel, *Anguilla japonica* (Nose and Arai, 1979) and 150 mg kg^{-1} in red sea bream, *Pagrus major* (Sakamoto and Yone, 1976). In addition, the dietary levels of 100–250 mg.Fe kg.diet^{-1} have been reported to supply salmonids the appropriate nutritional requirements of this mineral (Desjardins *et al.*, 1987; Andersen *et al.*, 1996). All these studies show that the dietary iron requirement of fish is species-specific and should be optimized for each cultured fish. Such data is unavailable for ship sturgeon. Ship sturgeon is ecologically and commercially one of the most important chondrostei fish of the Caspian Sea. This species was listed as a critically endangered fish due to depleting populations of them in the nature (Gesner *et al.*, 2010). Thus, the artificial propagation and pond culture of ship sturgeon is ongoing in some fish culture facilities of Iran. In culture conditions, attention to fish health and welfare especially in relation to nutritional factors is essential to enhance fish aquaculture in controlled conditions. Similar to other vertebrates, blood parameters are used as indices of health status in fish. One of the main factors influencing these parameters is nutrition. Therefore, the aim of the present experiment was to study the

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effects of different levels of iron on health status of ship sturgeon by assessing some haematological parameters (as indices of health status) including RBC, WBCs, differential WBCs, Hct, Hb, MCH and MCHC, MCV, IgM, ferritin, transferrin and plasma Fe^{2+}

Materials and methods

225 ship sturgeon juveniles (19.9±0.7 g) were obtained from Dr. Dadman International Sturgeon Research Institute, Rasht, Iran. Fish were distributed in 15 tanks containing 350 lit aerated (2.5 L air min^{-1}) and dechlorinated freshwater with 15 fish per tank. Totally, 4 experimental treatments and one control group with three replicates were considered. The experimental treatments were fish fed basic diets containing different levels of $FeSO_4$ including: Control group: 0 mg.$FeSO_4$ kg^{-1}.diet, T_1: 100 mg.$FeSO_4$ kg.$diet^{-1}$, T_2: 200 mg.$FeSO_4$ kg.$diet^{-1}$, T_3: 250 mg.$FeSO_4$ kg.$diet^{-1}$, T_4: 300 mg.$FeSO_4$ kg.$diet^{-1}$. The general composition of experimental basic diets is presented in Table 1. In addition to iron, a vitamin and mineral premix was added to 1 kg basic diet. The composition of these premixes were: (a) vitamin premix: Vit A: 16000000 IU; Vit D_3: 400000 IU; Vit E: 40g; Vit k_3 (K-stab): 2g; Vit B_1 (Thiamin): 6g; Vit B_2 (Riboflavin): 8g; Vit B_3 (Ca-Pantothenate): 12g; Vit B_5 (Niacin): 40g; Vit B_6 (Pyridoxine): 4g; Vit B_9 (Folic acid): 2g; Vit B_{12} (Cyanocobalamin): 8g; Vit H_2: 0.24g; Vit C: 60g; Inositol: 60g; B.H.T: 20g. (b) Mineral premix: Choline chloride %60: 6000mg; MnO_2 %45: 5004 mg; ZnO_2 %80: 9800 mg; $CuSO_4$ %20:600 mg; $CoSO_4$ %18: 100 mg; CaI_2 %60: 600 mg; Na_2Se %1: 20 mg; perlite; zeolite; $CaCO_3$. During the 60 day experiment, fish were fed experimental diets three times a day at 3% of body weight. Also, water quality parameters were monitored daily. During 60 days of the experiment, the water temperature was 20.1±1.9 °C, dissolved oxygen was 8.5±0.3 mg L^{-1} and pH was recorded as 7.8±0.1.

Table 1: The general composition of basic diets used in the present study.

Total protein (%)	Total fat (%)	Moisture (%)	Ash (%)	Energy content (Kcal kg^{-1})
40.08±0.12	13±0.82	9.48±0.45	8.6±0.94	4566.85±1.69

Assessment of hematological parameters

After 60 days of the experiment, blood samples (1 mL from each fish) were taken from the caudal vein of fish using a heparinized syringe. 0.2 mL of each sample was allocated to assess the haematological parameters of serum (RBCs, WBCs, Hct, Hb, MCV, MCH, MCHC) and the remainder was centrifuged (3000 g, 10 min) to separate serum for further analysis (IgM, total protein, ferritin, transferrin and Fe^{2+}). The MCV, MCH and MCHC values were calculated as follows:

MCV (fl)=(haematocrit value)/total number of RBCs (million mm^{-3})×10

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MCH (pg)=(haemoglobin concentration)/ total number of RBCs (million mm^{-3})×10

MCHC (%)=(haemoglobin concentration) / (haematocrit value)×100

Hct values were determined using the microhaematocrit capillary tubes according to Klontz (1994). The Hb values were determined by Cyanmethemoglobin according to Klontz (1994). RBCs, WBC and differential WBCs were determined with chamber method using Neubauers haemocytometer (Drabkin, 1945).

Total protein was measured photometrically according to Biuret-method (Hiller *et al.,* 1948; Thomas, 1998; Johnson *et al.,* 1999). The serum IgM and transferrin were measured nephelometrically by Binding Site Nephelometry kit as suggested by Zilva and pannall (1984). The serum ferritin was analysed by immunoradiometric assay (IRMA) technique (Flowers *et al.,* 1986). Serum ferritin was standardized according to the international standards included with the radioimmunoassay kit (Radim Co, Italy) and a gamma counter (Hewlett Packard, Wilmington, Del, USA). The Fe^{2+} concentration was determined by the photometric method with ferene (ferroin-type reagent) without deproteination (Higgins, 1981).

Statistical analysis

All data were analyzed by SPSS software. Data normality was investigated by Shapiro-Wilk test. Because percentage data did not have a normal distribution, proportional data were converted by angular transformation (arcsin √p). One-way analysis of variance (ANOVA) was employed to compare the means. When significant F-ratios were calculated by ANOVA, the Duncan test was applied to identify which means were different.

Results

The values of all parameters i.e. WBCs, lymphocytes, neutrophils, monocytes, eosinophils, MCH and MCHC, MCV, total plasma protein, IgM, ferritin, transferrin and plasma Fe^{2+} showed significant differences between experimental groups (Table 2) (p<0.05). Higher levels of IgM (10.10±2.13 mg dL^{-1}) and total plasma protein (2.27±0.12 g dL^{-1}) were observed in the 100 mg.$FeSO_4$ kg.$diet^{-1}$ treatment, compared with other experimental groups (Table 2) (p<0.05). The highest values of neutrophils (35.9±15.4), monocytes (1.33±0.5) and MCHC (23.33±2.87 %) were found in control fish (Table 1) whereas MCV (326.5±11.7 FL), MCH (63.75±2.49 pg) and eosinophils (3±1.2) values were higher in the 200 mg $FeSO_4$ kg.$diet^{-1}$ treatment (Table 2) (p<0.05). Also, more WBCs (10877±238.6) were observed in the 250 mg $FeSO_4$ kg.$diet^{-1}$ treatment than in other groups (Table 2) (p<0.05). The values of lymphocytes (68.1±1.45), RBC (1500444.4±117652.2), Hct (39±1.32 %), Hb (8.24±0.47 g dL^{-1}), ferritin (0.55±0.01 ng dL^{-1}), transferrin (203.7±3.79 mg dL^{-1}) and plasma Fe^{2+} (57.67±1.16 ng dL^{-1}) were higher in fish fed 300 mg $FeSO_4$ kg.$diet^{-1}$

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compared to other experimental groups (Table 2) (p<0.05).

Table 2: Haematological parameters of ship sturgeon fed by different levels of iron ($FeSO_4$). The values with different letters in the table are significantly different (p<0.05).

parameters	Experimental groups 0 (mg iron kg^{-1})	100 (mg iron kg^{-1})	200 (mg iron kg^{-1})	250 (mg iron kg^{-1})	300 (mg iron kg^{-1})
WBC (mm^3)	8388.9±850.7^{b}	5040±1910.3^{a}	10825±349.5^{c}	10877±238.6^{c}	10333.3±785^{c}
lymphocytes	59.4±1.81^{a}	59±1.76^{a}	63.2±1.17^{b}	66.4±1.42^{c}	68.1±1.45^{d}
neutrophils	35.9±15.4^{c}	36.5±1.84^{c}	32.4±1.51^{b}	31.7±1.66^{b}	30±1.58^{a}
monocytes	1.33±0.5^{b}	1.3±0.68^{b}	1.13±0.64^{b}	0.56±0.53^{a}	0.44±0.53^{a}
eosinophils	3.22±0.97^{b}	3.2±1.55^{b}	3±1.2^{b}	1.33±0.5^{a}	1.44±0.73^{a}
RBC (mm^3)	478555.6±66940.5^{a}	1007100±59759.6^{b}	1105250±171782.9^{c}	1174111±58040.4^{c}	1500444.4±117652.2^{d}
Hb(g dL^{-1})	2.93±0.34^{a}	6.26±0.41^{b}	6.65±0.08^{c}	7.12±0.32^{d}	8.24±0.47^{e}
Hct (%)	12.8±0.97^{a}	31.8±1.62^{b}	34±0.54^{c}	35.2±0.83^{d}	39±1.32^{e}
MCV (FL)	267.8±33.03^{a}	316.2±11.9^{bc}	326.5±11.7^{c}	300.4±9.26^{b}	262.6±16.19^{a}
MCH(Pg)	62.56±11.2^{b}	62.2±1.4^{b}	63.75±2.49^{b}	60.44±0.73^{b}	55±2.29^{a}
Table 2 continued:					
MCHC(%)	23.33±2.87^{b}	19.8±0.79^{a}	19.63±0.52^{a}	2.33±0.5^{a}	21±0.71^{a}
Total protein (g dL^{-1})	1.8±0^{c}	2.27±0.12^{d}	1.7±0^{c}	1.5±0.1^{b}	0.57±0.14^{a}
IgM (mg dL^{-1})	5.17±0.5^{b}	10.10±2.13^{c}	4.85±0.21^{b}	3.37±0.46^{ab}	1.70±0.2^{a}
Iron (ng dL^{-1})	29.67±1.53^{a}	44.67±2.08^{b}	49.5±0.71^{c}	54.67±1.16^{d}	57.67±1.16^{e}
Ferritin(ng dL^{-1})	0.29±0.03^{a}	0.39±0.02^{b}	0.45±0.06^{c}	0.51±0.02^{d}	0.55±0.01^{e}
Transferrin (mg dL^{-1})	143.7±1.52^{a}	165±4.58^{b}	174.5±4.95^{b}	186±8^{c}	203.7±3.79^{d}

Discussion

The changes in haematological components reflect the health status of fish in relation to various internal and external factors including environmental conditions and nutrition (Houston, 1997; Osuigwe *et al.*, 2005). The quality of the food affects haematological parameters of sturgeons (Domezain *et al.*, 1999). In the present study, the effects of dietary levels of iron were investigated on haematological components of ship sturgeon for the first time. More values of RBC, Hct, Hb, ferritin, transferrin and plasma Fe^{2+} were observed when fish were fed 300 mg.iron $kg.diet^{-1}$. Thus, it seems that the dietary level of 300 mg.iron $kg.diet^{-1}$ is appropriate and adequate for the formation of haemoglobin and RBCs. This assertion could be supported by higher levels of plasma iron in 300 mg.iron $kg.diet^{-1}$ treatment compared to other groups since iron is an essential element for the formation of haemoglobin and RBC (Roeder and Roeder, 1966; Aisen *et al.*, 1972; Walker and Fromm, 1976; Bernat, 1983; Desjardins *et al*, 1987; Bjornvic and Maage, 1993; Andersen *et al.*, 1996). Similar to our results, increases in RBC, Hct and Hb in fish fed Fe supplemented diets was reported in some fish species (Roeder and Roeder 1966; Gatlin and Wilson, 1986; Lim *et al.*, 1996; Andersen *et al.*, 1997; Lim *et al.*, 2000; Carriquiriborde *et al.*, 2004; Pan *et al.*, 2009). However, some studies have reported no changes in RBC, Hct and Hb in response to various levels of dietary iron which may be species-specific (Maage and Sveier, 1998; Vangen and Herme, 2003; Pan *et al.*, 2009; Aride *et al.*, 2010; Rigors *et al.*, 2010).

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In our study, the plasma levels of iron increased as its dietary levels elevated. Similar results have been reported in other fish species (Gatlin and Wilson, 1986; Chen and Shiau, 2005; Ling *et al.*, 2010). On the whole, iron is not free in the circulation but transported as transferrin and ferritin compounds (bound to protein). These compounds store iron and release it in a controlled fashion (Harper, 1975). In our study, the values of transferrin and ferritin were higher in the 300 mg.iron kg. diet^{-1} treatment than in other groups which may be related to the higher concentration of plasma iron in this treatment that needs to be carried in the plasma. This case could be supported when we previously observed the higher concentration of iron in the 300 mg.iron kg^{-1} diet treatment. In a study by Carriquiriborde *et al.* (2004), the increase in total serum iron from 10 to 49 µmol L^{-1} over 8 weeks was associated with elevated total Fe binding capacity and decreased unsaturated Fe binding capacity, so that in fish fed a high Fe diet transferrin saturation increased from 15% at the start of the experiment to 37%. Also, in Channel catfish, the elevation of plasma iron coincided with increases in plasma transferrin and ferritin (Lim and Klesius, 1997). In our study, lymphocytes composed the highest percentage of WBCs as reported for stellate sturgeon, *Acipenser stellatus* (Yosefi Jurdehi, A., 2006). A reverse relationship between lymphocyte numbers and fish weight was reported by Orun *et al.* (2003) as we observed the lowest values of lymphocytes in ship sturgeons fed 100 mg. iron kg.diet^{-1} which had the highest body weight after 60 days. The same authors showed a positive relationship between fish weight and the number of neutrophils and monocytes. This result was confirmed in our study when higher levels of neutrophils and monocytes were observed in fish which showed a higher body weight and were fed diets containing100 mg.iron kg.diet^{-1}. In the present study, higher WBC counts were observed in treatments fed diets containing 250 and 300 mg.iron kg.diet^{-1} compared to other experimental groups. These results show that the immune system of ship sturgeon juveniles might be enhanced by diets containing high iron supplements. Palikova *et al.* (1999) suggested nutrition as the main parameter influencing the haematological indices in fish. However, the results of IgM concentrations presented different trends compared to WBCs where IgM levels (as one of the main responses of fish immune system) were higher in the 100 mg.iron kg.diet^{-1} treatment than in other groups. On the other hand, the values of lymphocytes were lower in the 100 mg. iron kg.diet^{-1} treatment. Lymphocytes including T-Cells and B-Cells (responsible for IgM production) are the main cells involved in adaptive immunity of fish (Uribe *et al.*, 2011). It seems that the stimulatory action of iron in fish fed diets containing 100 mg kg^{-1} iron is modulated more through B-Cells and subsequent production of IgM. In addition to B-Cells, probably the level of iron in the diet with 100 mg.iron kg.diet^{-1} stimulates more innate

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immunity components compared to the higher levels of dietary iron. In this regard, innate immunity components including neutrophils, monocytes and eosinophils were higher in the 100 mg.iron kg.diet^{-1} treatment than in the 250 and 300 mg Fe.iron kg.diet^{-1} treatments. In contrast, the higher levels of dietary iron (250 and 300 mg.iron kg.diet^{-1} treatments) probably affect the immunity of ship sturgeon through the higher number of T-Cells since the lower levels of IgM were observed in fish fed diets with 250 and 300 mg.iron kg.diet^{-1}. In this study, the total plasma protein decreased as the dietary levels of iron increased which may be related to the participation of proteins in the structure of haemoglobin and formation of RBCs (Bernat, 1983). Also, the lowest values of MCV and MCH were found in fish fed 300 mg.iron kg.diet^{-1}, which may be due to the high levels of RBCs in this treatment. In conclusion, the results of the present study suggested that supplementation of ship sturgeon diets with high levels of iron enhances the RBC, Hct, Hb, ferritin, transferrin and plasma iron. However, the effects of dietary iron on immunity of this species are different depending on the levels used in the diet. At low dietary levels, iron stimulates more innate immunity components whereas at high levels the adaptive immunity is influenced more.

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