

# Nano-Fe as feed additive improves the hematological and immunological parameters of fish, *Labeo rohita* H.

T. Behera · P. Swain · P. V. Rangacharulu · M. Samanta

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**Abstract** An experiment was conducted to compare the effects of iron oxide nanoparticles ( $T_1$ ) and ferrous sulfate ( $T_2$ ) on Indian major carp, *Labeo rohita* H. There were significant differences ( $P < 0.05$ ) in the final weight of  $T_1$  and  $T_2$  compared with the control. Survival rates were not affected by the dietary treatments. Fish fed a basal diet (control) showed lower ( $P < 0.05$ ) iron content in muscle compared to  $T_1$  and  $T_2$ . Furthermore, the highest value ( $P < 0.05$ ) of iron content was observed in  $T_1$ . In addition, RBCs and hemoglobin levels were significantly higher in  $T_1$  as compared to other treated groups. Different innate immune parameters such as respiratory burst activity, bactericidal activity and myeloperoxidase activity were higher in nano-Fe-treated diet ( $T_1$ ) as compared to other iron source ( $T_2$ ) and control in 30 days post-feeding. Moreover, nano-Fe appeared to be more effective ( $P < 0.05$ ) than ferrous sulfate in increasing muscle iron and hemoglobin contents. Dietary administration of nano-Fe did not cause any oxidative damage, but improved antioxidant enzymatic activities (SOD and GSH level) irrespective of different iron sources in the basal diet.

**Keywords** Fish · Feed · Iron · Nano-Fe · Immune response · *Labeo rohita*

## Introduction

Iron (Fe) is an indispensable element for the functioning of organs and tissues of higher animals, including fish, because of its vital role in physiological processes such as oxygen transport, cellular respiration and lipid oxidation reactions (Lee et al. 1981; Andersen et al. 1997). Iron is also one of the most essential micronutrients in terms of its effect on the functioning of the immune system and defense against various infections (Beisel 1982; Bhaskaram 1988). Iron deficiency causes immune suppression, growth depression, changes in hematological parameters, susceptibility to diseases, poor food conversion and microcytic anemia in common carp (Tacon 1992; Andersen et al. 1996; Kawatsu 1972; Sakamoto and Yone 1978). Iron is thus essential and in excess can be toxic (Salte et al. 1994; Andersen et al. 1997). Fish can absorb soluble iron from the water across the gill membrane and intestinal mucosa (Roedar and Roedar 1966; Sealey et al. 1997). However, feed is considered to be a major source of iron for fish, due to low concentration of soluble iron in natural water and limited iron passage across the gills (NRC 1993; Bury et al. 2003). As in higher vertebrates, the mechanisms involved in the absorption and regulation of iron in fish are poorly understood (Lall 2002). Till now several reports showed contradictory results for the dietary iron requirements in fish, which vary from species to species and in different environmental conditions (Hem 1989). The minimum dietary iron requirement in Indian major carp, rohu, *Labeo rohita* H. was 0.014 % of dry diet (Jhingran and Pullin 1985).

Different chemical forms of iron exhibit different bioavailabilities in animals (Forbes et al. 1989); however, this information is lacking in fish (Lall 2002). Most of the chemical forms of iron have poor bioavailability to fish.

T. Behera · P. Swain (✉) · M. Samanta  
Fish Health Management Division, Central Institute  
of Freshwater Aquaculture, Kausalyaganga,  
Bhubaneswar 751 002, Orissa, India  
e-mail: truptibiotech2007@gmail.com

P. V. Rangacharulu  
Fish Nutrition and Physiology Division, R.R.C. Central Institute  
of Freshwater Aquaculture, Vijayawada, Andhra Pradesh, India

However, ferrous fumarate ( $C_4H_2FeO_4$ ), ferrous sulfate ( $FeSO_4 \cdot 7H_2O$ ) and heme iron are highly bioavailable than other water-insoluble elemental and non-heme form of iron (Melodie 2011; Coffman 2011), whereas ferric phosphate nanoparticles ( $FePO_4$ ) have low nutritional value due to their low bioavailability (Rohner et al. 2007). At present, not a single evidence showed iron sulfide is bioavailable to fish. Using a bioavailable form, dietary iron will improve the quality of fish feed for the aquaculture industry. Different forms of iron cause unacceptable changes in the color or taste of foods, are poorly water-soluble, cause sensory changes and are not well absorbed. Iron oxides, on the other hand, have better sensory performance than phosphates, but are poorly soluble and may have lower bioavailability (Hilty et al. 2011). Recently, a preliminary report showed that nanostructured iron oxide ( $Fe_2O_3$ ) could be as bioavailable as ferrous sulfates ( $FeSO_4$ ) in rats (Hilty et al. 2011). Different chemical forms of iron have different degrees of limitations to fulfilling the dietary iron requirements in fish. Determining the optimum iron feed concentration and its suitable bioavailable form is therefore a necessary task for the performance of any farmed fish of interest. However, such data are limited particularly in teleost fish.

Nanotechnology holds promise for both medication and nutrition, because materials at the nanometer dimension exhibit novel properties different from those of isolated atom and bulk material (Albrecht et al. 2006; Wang et al. 2007). Iron oxide nanoparticles are of great interest due to their unique physicochemical properties and have a great potential in human biomedical applications, food additives, antimicrobial additives, drug carriers, etc. (Huber 2005). Moreover, food additives in the nano forms are being increasingly used including iron-fortified cereals and drinks for human consumption (Hilty et al. 2010a, b). Recently, preliminary reports showed that iron oxide nanoparticles could boost bioavailability than other forms of iron nanoparticles in both humans and rats through dietary administration (Stephen 2007). Iron is also associated with protein in animal tissues (Barton and Edwards 2000). Consequently, muscle protein and seafood are dependable dietary sources of the minerals (Gibson 1990). Deteriorative oxidative reactions in meat lead to losses of both nutritional value and food quality. To increase the oxidative stability and meat quality, antioxidants such as iron have been added to the feed of farm animals (Min et al. 2008).

However, not a single study has been done to incorporate nano-Fe into fish feed based on growth performance, hematology, immune response, tissue deposition and antioxidant enzymatic activities. Very little information is available regarding the mineral requirements of Indian major carps, *Labeo rohita*, but the same assumes great

importance in feed formulations. Under practical farming conditions, iron deficiencies occur mainly due to low bioavailable iron in commercial fish feeds. Therefore, in this study we evaluated the application of different iron sources, including iron nanoparticles (nano-Fe) and ferrous sulfate ( $FeSO_4 \cdot 7H_2O$ ), as feed additives in diets for, rohu, *Labeo rohita* H. which is one of the commercially important and valuable freshwater fish species cultured in India.

## Materials and methods

### Materials and diet

Iron oxide ( $Fe_2O_3$ ) nanoparticles (nano-Fe) (average size  $<50$  nm by TEM analysis, purity  $\geq 98$  % trace metals basis) and ferrous sulfate ( $FeSO_4 \cdot 7H_2O$ ) were purchased from Sigma-Aldrich, USA. The nano-Fe was in the redox state of zero. The desired amount of nano-Fe was diluted (evenly dispersed) with dechlorinated water to make up a suitable dose for oral administration through the feed. Commercial kits of superoxide dismutase (SOD) and glutathione assay (GSH) were purchased from Sigma-Aldrich, USA, and Quanti-Chrome assay Kits, respectively. Ingredients and chemical composition of the basal diets used in the study were according to Lovell (1998). Basal diet formulations are shown in Table 1. The analysis of the iron concentrations in the experimental diets shows the approximate values of the expected concentration as given in Table 1.

**Table 1** Formulations of experimental diets

Ingredients	(%)
Casein	32
Gelatin	8
Dextrine	28
Cellulose	19
Fat <sup>a</sup>	6
Carboxy methyl cellulose	2
Mineral premix <sup>b</sup>	4
Vitamin premix <sup>c</sup>	1

<sup>a</sup> The mixture of fish oil and lard (1:1)

<sup>b</sup> Mineral premix (%):  $KAl(SO_4)_2$ , 0.159;  $CaCO_3$ , 18.101;  $Ca(H_2PO_4)_2$ , 44.601;  $MgSO_4$ , 5.216;  $CoCl_2$ , 0.07;  $KCl$ , 16.553;  $Na_2SeO_3$ , 1.338;  $MnSO_4 \cdot H_2O$ , 0.07;  $KI$ , 0.014;  $ZnSO_4$ , 0.192;  $NaH_2PO_4$ , 13.605; and  $CuSO_4 \cdot 5H_2O$ , 0.075

<sup>c</sup> Vitamin premix: thiamin hydrochloride, 10 mg  $kg^{-1}$ ; riboflavin, 20 mg  $kg^{-1}$ ; calcium pantothenate, 40 mg  $kg^{-1}$ ; nicotinic acid, 50 mg  $kg^{-1}$ ; pyridoxine hydrochloride, 10 mg  $kg^{-1}$ ; folic acid, 5 mg  $kg^{-1}$ ; inositol, 400 mg  $kg^{-1}$ ; choline chloride, 2,000 mg  $kg^{-1}$ ; menadione, 10 mg  $kg^{-1}$ ; cholecalciferol, 1,500 IU; biotin, 1 mg  $kg^{-1}$ ; vitamin  $B_{12}$ , 0.02 mg  $kg^{-1}$ ; vitamin A, 3,000 IU; vitamin E, 50 IU; and vitamin C, 200 mg  $kg^{-1}$

## Diet formulations

Six tanks with duplicates for treatments and control were used. Three treatments were used. In treatment 1 ( $T_1$ ) and treatment 2 ( $T_2$ ), nano-Fe and ferrous sulfate (best bio-available and soluble iron supplement) were added to the experimental basal diets at the rate of  $0.5 \text{ mg kg}^{-1}$  dry feed weight, respectively. The control groups included only basal diet without iron supplements. The ingredients and different iron sources were mixed, extruded and air dried at room temperature. Then these diets were kept at  $-20 \text{ }^\circ\text{C}$  until use. To mix adequately, nano-Fe or ferrous sulfate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) was slowly applied to the diet ingredients, mixing part by part in a mixer. The final actual concentration of iron in each diet was determined and is shown in Table 2.

## Fish and experimental design

Indian major carp, *Labeo rohita* (rohu), juveniles having similar average weight (Table 3) were acclimatized in the wet laboratory of Fish Health Management Division of Central Institute of Freshwater Aquaculture (CIFA), Kausalyaganga, India, 30 days prior to the start of the experiment. Water temperature of the experimental tanks was  $27\text{--}30 \text{ }^\circ\text{C}$  with a photoperiod of 12-h light and 12-h darkness. The tanks were supplied with running freshwater. For water quality control, temperature and dissolved oxygen (DO) were measured daily, and weekly analyses were conducted for total ammonium, nitrite and pH levels using the kit.

**Table 2** The actual concentration of iron in all experimental diets

Group	Control	$T_1$	$T_2$
Iron sources	–	Nano-Fe	Ferrous sulfate
Actual concentration ( $\text{mg kg}^{-1}$ )	$0.05 \pm 0.0005$	$0.54 \pm 0.002$	$0.55 \pm 0.0009$

**Table 3** Growth performance and survivability of *Labeo rohita* H. supplemented with different iron sources (nano-Fe and ferrous sulfate,  $T_1$  and  $T_2$ , respectively) and without iron (control)

Group/treatment	$T_1$	$T_2$	Control
Initial weight (g)	$20.22 \pm 0.44^a$	$20.29 \pm 0.68^a$	$20.45 \pm 0.50^a$
Final weight (g)	$38.07 \pm 0.9^a$	$36.0 \pm 0.0^a$	$28.25 \pm 0.88^b$
Survival rate (%)	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>

Results are presented as mean  $\pm$  SE of triplicate observations. Means bearing different superscripts were significantly different ( $P < 0.05$ )

Then the fish were distributed into six tanks at 20 fish per tank with similar initial weights. The experiment was conducted as a completely randomized design with three treatments ( $T_1$ ,  $T_2$  and Control). Daily (twice a day) feeding rate was about 3 % of total body weight and care was also taken to ensure that all the feed added to the tanks was eaten. Every day, the diet remaining in each tank was collected by siphoning before the second day's feeding. A daily record was kept of feed offered and the remains. Every day, each tank was cleaned with 50 % water being changed.

## Feed and tissue analysis

Feed and tissue (gill, muscle and liver) samples were dried. One gram of dried tissue samples was digested separately with 10 ml of  $\text{HNO}_3$  in a microwave device (CEM MDS 2100). Following digestion, the sample was diluted with distilled water to 20 ml and filtered. A Varian AA 20 Plus flame atomic absorption spectrophotometer was used for the determination of the metal concentrations.

## Hematological parameters

Different hematological parameters were studied 30 days post-feeding (dpi) in all the treated groups. Red blood cells (RBCs) and white blood cells (WBCs) diluting fluids were used for determining total erythrocyte and leucocyte counts. It was done by mixing 20  $\mu\text{l}$  of blood with 3,980  $\mu\text{l}$  of the corresponding diluting fluid in a clean test tube. Cell counts were performed using a Neubauer's counting chamber. The hemoglobin level of blood was analyzed following the cyanomethemoglobin method using Drabkins Fluid (Qualigens Chemicals) (Darbkin 1950). The absorbance was measured using a spectrophotometer at 540 nm and the final concentration was calculated by comparing with the standard cyanmethemoglobin (Qualigens Chemicals). The hemoglobin concentration was then calculated using the following formula: hemoglobin (g/dl) =  $[\text{OD (T)}/\text{OD (S)}] \times [251/1,000] \times 60$  where OD (T) is the absorbance of the test and OD (S) the absorbance of the standard. Serum protein was estimated with a BCA protein estimation kit (total protein) (Bangalore Genei) and albumin by albumin estimation kit (Qualigens Diagnostics, Glaxo smithkline). Albumin was estimated by the bromocresol green binding method (Doumass et al. 1971). The absorbance of the standard and test was measured against a blank in a spectrophotometer at 630 nm. Globulin was calculated by subtracting albumin values from total serum protein. A/G ratio was calculated by dividing albumin values by globulin values.

### Innate immune parameters

Different innate immune parameters were studied in all the experimental groups at 30 dpi.

### Respiratory burst activity

The respiratory burst activity of the phagocytes was performed using nitroblue tetrazolium (NBT) assay following the method of (Anderson and Siwicki 1994). Blood (50  $\mu$ l) was placed into the wells of U-bottom microtiter plates and incubated at 37 °C for 1 h to allow adhesion of cells. Then the supernatant was removed and the wells were washed three times in PBS. After washing, 50  $\mu$ l of 0.2 % NBT was added and incubated for a further 1 h. The cells were then fixed with 100 % methanol for 2–3 min and washed three times with 30 % methanol. The plates were air dried, and 60  $\mu$ l 2 N potassium hydroxide and 70  $\mu$ l dimethyl sulfoxide were added to each well. The OD (optical density) was recorded in an ELISA reader (BIORAD-USA) at 540 nm.

### Serum bactericidal activity

The serum was assayed for bactericidal activity as described by Rainger and Rowley (1993). For this, *Aeromonas hydrophila* culture was centrifuged and the pellet washed and suspended in PBS. The optical density (OD) of the suspension was adjusted to 0.65 at 540 nm. This bacterial suspension was serially diluted (1:10) with PBS. Serum bactericidal activity was determined by incubating 2  $\mu$ l of the diluted *A. hydrophila* cell suspension with 20  $\mu$ l of serum in a micro-vial for 1 h at 37 °C. In the control group, PBS was used in the place of serum. After incubation, the number of viable bacteria was determined by counting the colonies grown on nutrient agar plate for 24 h at 37 °C.

### Alternative complement activity (ACH<sub>50</sub>)

The determination of alternative serum complement activity was done according to the previous method with little modifications (Sitja-Bobadilla et al. 2003). Rabbit red blood cells (RRBCs,  $2.5 \times 10^8$  cells ml<sup>-1</sup>) were taken from blood. One hundred microliters of diluted serum (1:25 in HBSS solution containing 10 mM Mg<sup>2+</sup> and 10 mM ethylene glycol bis tetraacetate, pH 7.6) was taken in Eppendorf tubes and mixed with 25  $\mu$ l of RRBCs. The tubes were incubated for 90 min at 150 rpm at 20 °C and then centrifuged to spin down the remaining RRBCs. The supernatants were transferred into 96-well plates and measured at 415 nm. The serum dilution factor was plotted in logarithmic scale against the percentage of RRBCs lysed at each dilution. The dilution corresponding to 50 % hemolysis ml<sup>-1</sup> was expressed as ACH<sub>50</sub>.

### Oxidative stress and enzymatic activities

Liver tissues were collected in each group separately at 30 days post-feeding, homogenized with ice-cold 0.86 % NaCl and centrifuged at 12,000 $\times$ g at 4 °C. The resulting supernatants were used for the determination of lipid peroxidation by MDA assay, total glutathione (GSH) and superoxide dismutase (SOD) activities using commercial kits (SOD assay kit, Sigma; QuantiChrom™ Glutathione (GSH) Assay Kit) and malondialdehyde (MDA) assay using the thio-barbituric acid technique (Ohkawa et al. 1979). The activities of SOD, GSH and MDA were measured as per the calculation given in the kit and the protocol.

## Results

### Water quality and growth performance

There was no adverse effect of different iron sources on water quality. Total ammonium (0–0.2 mg l<sup>-1</sup>), nitrite (0–0.1 mg l<sup>-1</sup>) and pH (7.4–7.8) were stable and within acceptable ranges (Boyd and Tucker 1998). At the beginning, no significant difference was observed in the initial weight between T<sub>1</sub>, T<sub>2</sub> and the control (Table 3). At the end of experiment (after 30 days of culture), there were significant differences ( $P < 0.05$ ) in relative weight gain and final weight of T<sub>1</sub> and T<sub>2</sub>, as compared to the control. However, no significant differences ( $P < 0.05$ ) in relative weight gain and final weight between T<sub>1</sub> and T<sub>2</sub> was found (Table 3). Survival rates were not affected by the administrations of dietary iron in the whole experimental period.

### Iron concentration in serum and muscle tissues

After 30 days of culture, fish fed a nano-Fe diet (T<sub>1</sub>) showed a higher ( $P < 0.05$ ) iron content in the muscle tissue as compared to T<sub>2</sub> and control (Table 4). However, T<sub>2</sub> showed higher iron content ( $P < 0.05$ ) than the control in muscle tissues. Serum iron content was also significantly higher ( $P < 0.05$ ) in both T<sub>1</sub> and T<sub>2</sub> groups as compared to control. However, there was no significant difference ( $P > 0.05$ ) between the groups T<sub>1</sub> and T<sub>2</sub> (Table 4).

**Table 4** Iron contents in serum and muscles

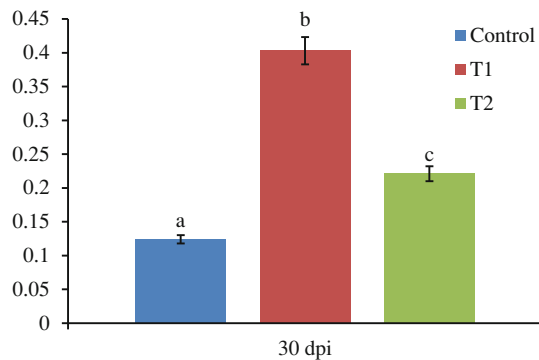
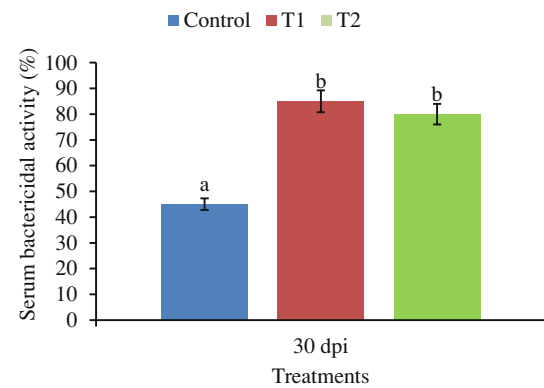
	Control	T <sub>1</sub>	T <sub>2</sub>
Serum iron (mg l <sup>-1</sup> )	0.98 $\pm$ 0.3 <sup>a</sup>	3.0 $\pm$ 0.5 <sup>b</sup>	3.01 $\pm$ 0.2 <sup>b</sup>
Muscle ( $\mu$ g g <sup>-1</sup> )	3.4 $\pm$ 0.75 <sup>a</sup>	5.8 $\pm$ 0.8 <sup>b</sup>	5.0 $\pm$ 0.16 <sup>c</sup>

Results are presented as mean  $\pm$  SE of triplicate observations. Means bearing different superscripts were significantly different ( $P < 0.05$ )

**Table 5** Hematological parameters and total serum proteins of *Labeo rohita* H. supplemented with iron oxide nanoparticles (nano-Fe,  $T_1$ ), ferrous sulfate ( $T_2$ ) and without any iron (control) in the basal diet

	RBC ( $10^6$ cells/ $\mu$ l)	WBC ( $10^3$ cells/ $\mu$ l)	Hemoglobin (g/dl)	Total protein (g %)	Albumin (g %)	Globulin (g %)
Control	$1.60 \pm 0.15^a$	$102.38 \pm 0.72^a$	$7.13 \pm 0.10^a$	$3.61 \pm 0.02^a$	$1.23 \pm 0.01^a$	$2.38 \pm 0.02^a$
$T_1$	$2.92 \pm 0.02^b$	$102.50 \pm 0.16^a$	$11.53 \pm 0.11^b$	$4.70 \pm 0.00^b$	$1.12 \pm 0^b$	$3.59 \pm 0^b$
$T_2$	$2.61 \pm 0.02^c$	$100.45 \pm 1.55^a$	$10.40 \pm 0.16^c$	$4.63 \pm 0.01^b$	$1.15 \pm 0^b$	$3.49 \pm 0.02^b$

Means bearing different superscripts were significantly different ( $P < 0.05$ )

**Fig. 1** Effect of dietary nano-Fe on respiratory burst activity in blood of *L. rohita* H. Values are the mean  $\pm$  SE. Means bearing different superscripts are statically significant ( $P < 0.05$ )**Fig. 2** Effect of dietary iron on serum bactericidal activity of *L. rohita* H. Values are the mean  $\pm$  SE. Means bearing different superscripts are statically significant ( $P < 0.05$ )

### Hematological effect

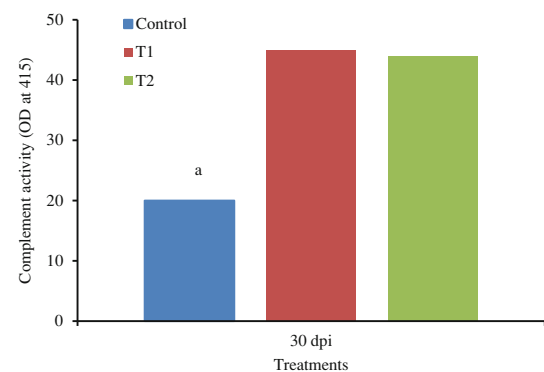
Hematological analysis showed significantly higher levels ( $P < 0.05$ ) of RBCs and hemoglobin content in group  $T_1$  and  $T_2$  diets as compared to the control diet (Table 5). Moreover, these values were significantly higher ( $P < 0.05$ ) in nano-iron diet ( $T_1$ )-treated group than in the  $T_2$  group. However, WBCs content, total serum protein, albumin, globulin and albumin/globulin ratio showed no significant differences among all the treated groups, such as  $T_1$ ,  $T_2$  and control diet.

### Immunological studies

Respiratory burst activity and serum bactericidal activity were significantly increased ( $P < 0.05$ ) in both iron treatment groups ( $T_1$  and  $T_2$ ) as compared to control (Figs. 1 and 2, respectively) after 30 dpi. However, there is a significant higher ( $P < 0.05$ ) respiratory burst and serum bactericidal activities in nano-iron-treated group ( $T_1$ ) as compared to the  $T_2$  group. However, there were no significant differences in alternative complement activity among  $T_1$ ,  $T_2$  and the control (Fig. 3).

### Antioxidant enzymatic activities and lipid peroxidation analysis

The potential impact of nano-Fe on antioxidant enzymatic activities and lipid peroxidation (SOD, GSH and MDA)

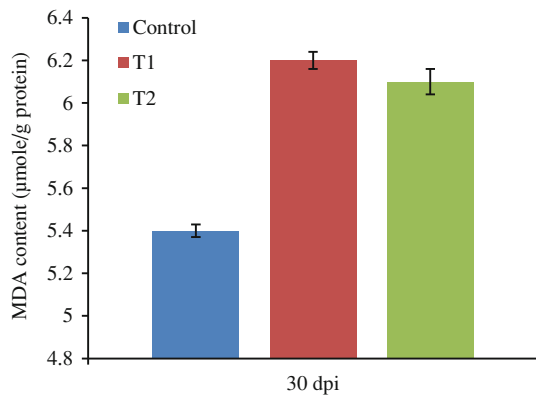
**Fig. 3** Effect of dietary iron on serum complement activity of *L. rohita* H. Values are the mean  $\pm$  SE. Means bearing different superscripts are statically significant ( $P < 0.05$ )

was investigated by taking liver tissues of all the treated groups after 30 days of culture. Lipid peroxidation measured by MDA assays (Fig. 4) showed no significant difference in both  $T_1$  and  $T_2$  as compared to control. In addition, both GSH (Fig. 5) and SOD (Fig. 6) activities were significantly higher in both  $T_1$  and  $T_2$  compared with the control. However, both SOD and GSH showed no significant difference between  $T_1$  and  $T_2$ .

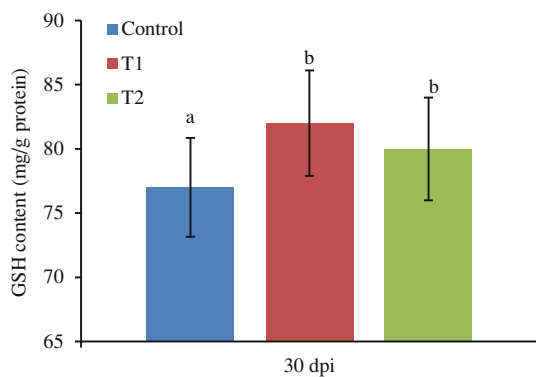
### Discussion

Dietary iron supplementation is necessary and Roeder and Roeder (1966) demonstrated that the growth of fish was

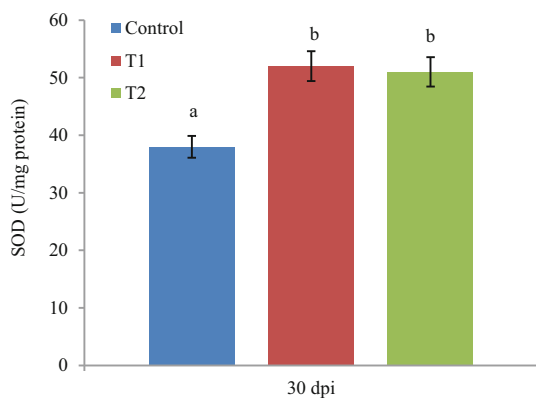




**Fig. 4** Lipid peroxidation activities (MDA assays) in liver tissues of *L. rohita* H. after 30 dpi



**Fig. 5** GSH activities in liver tissues of *L. rohita* H. after 30 dpi



**Fig. 6** SOD activities in liver tissues of *L. rohita* H. after 30 dpi

affected by dietary iron level. Little is known about iron metabolism in fishes. To our knowledge, this is the first report investigating the potential nutritional and immunological value of iron nanoparticles in fishes. According to Roeder and Roeder (1966), addition of ferrous sulfate to water in which fishes were raised appeared to have a stimulatory effect on the growth of these fishes, as compared to untreated controls (Roeder and Roeder 1966).

It clearly indicated that iron-supplemented diet could improve the final weight of Indian major carp, *Labeo rohita* H, whereas fishes showed growth retardation when given a purified diet without iron supplementation (Gatlin and Wilson 1986). Mortality noted in fishes fed an iron-deficient diet was prevented by administration of a diet containing optimum iron level. The nano-dispersed form of iron in feed drastically reduced mortality rate in commercial fish farms of carp and sturgeon (Prochorov et al. 2011). In this experiment, no mortality was recorded in any of the iron-treated group. It was obvious that the iron contents in muscle were markedly changed with the addition of dietary iron and was significantly ( $P < 0.05$ ) higher in  $T_1$ . The results indicated that nano-Fe and ferrous sulfate had different metabolic pathways, although both the inorganic form crossed the intestinal barrier. Thus iron bioavailability depended not only on its absorption by the intestine, but also on its conversion to a biologically active form (Andersen et al. 1997). In support to the present study, the nano form of iron oxide ( $Fe_2O_3$ ) is highly bioavailable (96 % similarity with  $FeSO_4$ ) in rats without tissue accumulation (Hilty et al. 2010a, b nature). This shows that nano-Fe could have a special metabolism pathway and deposition mechanism in fish.

Serum iron is a traditional iron status parameter and has been used as an indicator for iron requirement in fish (Gatlin and Wilson 1986). Iron found in the serum is considered a function of iron entering and leaving the blood stream (Silva et al. 2004). In our study, serum iron was improved with nano-Fe-treated diet as compared to other iron sources. This suggested that nano-Fe supplementation significantly increased the absorption and bioavailability of iron. Moreover, less absorption in fish fed a basal diet, deficient in iron, suggested that iron supplementation was necessary for Indian major carps. Hematological assays may provide an index of the physiological status of fish. These parameters are more sensitive to iron supplementation and act as indicator of iron requirements (Chu et al. 2007). In this study, a significant increase in RBCs and hemoglobin was found in three nano-Fe-treated diets unlike use of other iron sources and iron-deficient diet. Similarly, fish receiving nano-disperse form of iron through diet had significantly enhanced erythropoiesis and hemoglobin level, and drastically reduced mortality rate, without any sign of polychromatic anemia (Prochorov et al. 2011). Several reports showed that a deficiency of iron may contribute to several immunodeficiency syndromes such as impaired T cell functioning, atrophy in lymph organs, etc. in both humans and animals (Farthing 1989). In this study, no immune suppression was observed in all the treated groups. A tendency for increased respiratory burst activity, myeloperoxidase activity and bactericidal activity was observed in the nano-Fe diet group. The in vitro effects of

magnetic iron oxide nanoparticles on dendritic cell activation (Goya et al. 2008) and enhanced production of interleukins, cytokines, through intravenous administrations in rat (Chen et al. 2011) support our findings. Iron deficiency also causes decreased myeloperoxidase activity, bactericidal activity, neutrophil functions and NBT reductions (Dhur et al. 1989; Farthing 1989; Oppenheimer and Hendrickse 1983; Scrimshaw and San Giovanni 1997). However, alternative complement activity was not significantly influenced by dietary nano-Fe administration in *Labeo rohita* H. Similarly, in *Atlantic salmon*, specific hemolytic complement activities were not influenced by the administration of dietary iron sources (both organic and inorganic form) up to 20 weeks (Andersen et al. 1998).

Iron has a number of biological functions in animals including fish. It is well known that dietary iron modulates the antioxidant defense system such as glutathione peroxidase (GSH) and superoxide dismutase (SOD) by preventing free radical production and lipid peroxidation (MDA). By monitoring several biomarkers such as SOD, GSH and MDA after dietary administration of nano-Fe, it was found that no oxidative damages were found. In contrast to this, Li et al. 2009 suggested that waterborne iron nanoparticle causes oxidative damages at the early exposure period in medaka embryo. However, no terminal oxidative damage occurred during the whole exposure period due to the self-recovering capacity of adult fish (Li et al. 2009). However, in the present study, iron supplemented in the basal diet could improve the SOD and GSH activity irrespective of different iron sources (nano-Fe and ferrous sulfate).

In conclusion, this research demonstrated that different iron sources (nano-Fe and ferrous sulfate) supplemented in the basal diet could improve the final weight and antioxidant enzymatic activities, and induce hematological and immunological parameters of Indian major carps, *Labeo rohita* H. Moreover, nano-Fe appeared to be more effective than ferrous sulfate in increasing muscle iron and hemoglobin content. Its promising role in raising hemoglobin level may be used in future to treat anemic fish. Despite these promising results, future studies are needed for a complete evaluation of its wide application in various species of fish.

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