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DIETARY EFFECTS OF IRON AND IRON OXIDE NANOPARTICLES ON GROWTH, MUSCLE COMPOSITION AND HEMATOLOGICAL ASPECTS OF *LABEO ROHITA*

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Key words:
Labeo rohita, Hematology, Iron oxide, Iron, Growth and Muscle composition.

ABSTRACT

Two hundred *Labeo rohita* fingerlings of uniform size (2-3g), irrespective of sex, were used to investigate the effects of dietary Fe and Fe_2O_3-NPs on growth, muscle composition and hematological aspects of fish. Fish maintained on 32% crude protein, conventional basal feed was supplemented with Fe & Fe_2O_3 nanoparticles (each at 0.6 & 0.8 mg/kg supplementation levels) to replicate groups of fish for 90 days. Morphometric characteristics i.e. wet body weight (g) and body length (cm) was recorded fortnightly. It was found that the weight gain, body length, SGR and PER were significantly improved in the fish fed on nano iron oxide supplemented diet (T_2). Protein and lipid nutrients retention in the body meat was improved in group of fish fed with nano iron supplemented diet (T_3). A significant increase in hemoglobin (Hb) content, red blood cell (RBC) count, white blood cell count (WBC) and hematocrit (Ht) value was noticed throughout the study period when compared to control group. From the results, it was concluded that Fe-NPs@0.6mg/Kg level is more suitable for *Labeo rohita* with great growth potential and improved muscle composition and hematology as compared to other levels of Fe and Fe_2O_3 NPs.

1. INTRODUCTION

Nano technologycan be defined as the accommodating and regulation of substances at the nano-scale, at magnitudes roughly between 1 and 100 nm, where characteristic phenomenon permit novel applications. When adapted into nano-size, the materials indicate exclusive chemical and physical properties (Khan *et al.,* 2019). Nanoparticles are considered to be reasonably well-accepted in the body of human and have major uses in magnetic detection, magnetic resonance imaging (MRI), and hyperthermia and drug delivery. They have used in magnetic resonance imaging as distinctive agents and can afford larger imaging capability owing to their greater half-life (Han *et al.,* 2019). Nanotechnology have capability for both nutrition and medication, because at the nm dimensions materials show innovative properties contrasted to the secluded atoms and unpackaged materials in size, shape, surface area to volume ratio and conductivity.

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Among the metal-oxide NPs, Iron oxide nanoparticles are broadly used in bioengineering, biomedical and clinical applications because to their greater catalytic properties and exclusive magnetic abilities (Vallabani *et al.,* 2018).Iron is the major essential micronutrient because of its influence on the effectiveness of immune system and also defense against several infections. It is vital element for the effectiveness of the tissues androgens' of higher class animals including fish because of its vigorous role in the biological processes including lipid oxidation reactions, cellular respiration and oxygen transport (Uzo *et al.,* 2019).

Fishes are the main protein source among aquatic life, providing 7% of entire protein supplies with 80% of the animal protein consumption. Per capita yearly fish consumption in Pakistan is around 1.9 kilogram which is lowermost contrasted to the advanced countries i.e. about 20kg(FAO, 2017). Pakistan's native carps like *Labeo rohita,Cirrhinus mrigala* and *Catla catla* are cultured in private and government sectors, also topmost the natural

aquatic systems (Hussain *et al.,* 2011).For the period of former twenty years cultivation of fish has been renowned as the endorsing world food industry (Yıldırım *et al.,* 2014).

The chief objectives of fisheries industry are, primarily to produce better quality fish and secondly, to augment growth. The feed that used for fish cultivation should be inexpensive, enhance immunity, growth and rigorous health of fish, to attain extreme benefits from the fish culture (Ringo *et al.,* 2016).Fisheries industry can be progressed by using nanoparticles in the fish feed.

Fish can absorb soluble iron from the water across the gill membrane but feed is considered to be the main source of iron for fish because of the limited concentration of soluble iron in water. Fish accept and take feed comprising NMs. At the nano-size dietary minerals can pass into the cells more freely than their larger complements. This hastens their process of integration into the fish and also augments the capability of fish to quickly absorb drugs such as vaccines, hormones and nutrients (Terova *et al.,* 2018).Deficiency of iron causes growth reduction, immune damage, changes in the hematological parameters, poor food conversion, microcytic anemia and vulnerability to diseases in common carp (Akbary *et al.,* 2019).

However, very little research has been done to incorporate nano forms of iron into fish feed based on hematology and muscle composition. Little information is present for the mineral desires of *Labeo rohita*, Indian major carps, but these have greatest significance in feed preparations. Under applied farming conditions, deficiencies of iron occur primarily because of low bio-available iron in marketable fish feeds. Therefore, in this study we assessed the applications of different nano forms of iron (nano-Fe & Fe2O3) as feed additives in feed for *Labeo rohita*, rohu which is commercially important and broadly cultured fish particularly in Indian subcontinent and generally in Asia (Iqbal *et al.,* 2014).

The necessary tasks of this study were to produce high quality fish with high muscle protein, and to produce fish with better immune functions by improving its hematology and to determine the optimum level of iron and iron oxide nanoparticles for fish feed.

2. MATERIALS AND METHODS

Experimental fish

For the execution of this feeding trial, a total of 200 *Labeo rohita* fingerlings were bought from fish seed hatchery, Satyana road, Faisalabad. Before the commencement of the experiment fingerlings were acclimatized to laboratory conditions for about 15 days, weaned on the control diet (32% crude protein) once daily on 4% live wet body weight (Allan and Rowland, 1992).

Feed ingredients and diet preparation protocol

The basic diet ingredients were sunflower meal, fish meal, gluten, wheat bran, rice bran, vitamin premixes and canola oil. These ingredients were purchased from local market and in laboratory they were grinded to acquire fine powder and were weighed out. To prepare the four experimental diets, both iron and iron oxide nanoparticles (>50nm) (0.6 and 0.8 mg/kg) were mixed in 1liter of de-chlorinated water per kg and used this water to prepare the dough (Lovell, 1989), whereas the control diet was maintained without nanoparticles supplementation. The dough was pelletized in a laboratory pelletizer fixed with 2 mm diameter and the pellets were collected in trays and dried at room temperature. Diet was formulated by linear formulation method using Win feed 2.6 (Win Feed (UK) Ltd., Cambridge, UK) (Table 1).

Experimental design

After acclimatization, fingerlings of uniform size (2-3g), irrespective of sex, were randomly selected and evenly distributed in 10 glass aquaria (90L×30W×45H with 29L water capacity) at 20 fingerlings per aquarium with two replicates. The experiment was conducted as a Complete Randomized Design (CRD) with one control and four treatments viz. T_1: Fe_2O_3-NPs 0.6; T_2: Fe_2O_3-NPs 0.8mg/kg;T_3: Fe-NPs 0.6 and T_4: Fe-NPs 0.8mg/kg of fish feed. The fingerlings were fed to apparent satiation once a day at 4% of live wet body weight. The water quality parameters i.e. pH, dissolved oxygen and temperature through YSI pro-series multi parameter professional plus meter were monitored. Dissolved oxygen (5ppm) was maintained by air pumps through capillary system (APHA, 1998).

Growth Studies

During the trial, on the basis of fortnight from control and treated groups morphometric characteristics of sampled fish (10) were recorded which include live wet body

weight and body length. Fish fingerlings growth was calculated according to the method followed by (Brown, 1957).

Increase in wet body weight (g)

Average body weight of 1st observed fortnight - Average body weight of next observed fortnight

Increase in net total body length (cm)

Average body length of 1st observed fortnight - Average body length of next observed fortnight

$$\textit{Condition factor (K)} = W \times 10^5 \div L^3$$

$$SGR = \frac{\ln(\text{Final wet body weight}) - \ln(\text{Initial wet body weight}) \times 100}{\text{Time duration (Days)} \times 100}$$

$$\textit{Weight gain} = \text{Final weight} - \text{initial weight}$$

$$\textit{Weight gain\%} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

$$PER = \frac{\text{Final body weight} - \text{Initial body weight}}{\text{Amount of protein fed}}$$

Analytical methods/ Calculations

A representative sample of feed and body meat were homogenized utilizing a motor and pestle and examined chemically by AOAC (1990) procedures: dry matter (DM) by oven drying at 105 °C; crude protein (CP) by microkjeldahl investigation, gross energy by using formula and electric furnace(Eyela-TMF 3100) for ash. Lipid was determined by chloroform methanol extraction technique (Bligh and Dyer, 1959) through 10454 soxtec system HTz.

Dry matter (DM)

$$\text{Moisture\%} = \frac{W_1 - W_2}{\text{Wt. of sample}} \times 100$$

Dry matter=100−moisture%

Crude protein analysis:

$$N_2\% = \frac{\text{Volume of 0.1N } (H_2SO_4) \text{ used} \times 0.001 \times 250}{\text{Weight of sample} \times 10} \times 100$$

$$\text{Crude protein percentage} = N_2\% \times 6.25$$

$$\text{Percentage of crude fat} = \frac{W_2 - W_1 \times 100}{\text{Wt. of sample}}$$

NFE %= 100 - (crude protein + fat + moisture)

GE (Kcal/g) = (5.64 × protein %) + (9.44 × lipids %) + (4.11 × NFE%) (Henken *et al.*, 1986)

$$\text{Ash} = \frac{\text{Weight of ash} \times 100}{\text{Wt. of sample}}$$

Study of hematological parameters

After providing the different concentrations of iron and iron oxide nanoparticles for 90 days, fish from control and treated group were sacrificed and blood was drawn from caudal vain using plastic disposable syringe. The collected blood sample was transferred into small vials, which were previously rinsed with heparin. The whole blood sample was used for the estimation of hematological aspects like hemoglobin, RBCs and WBCs count.HGB was determined with Drabkin's (Drabkin, 1945) cyanmet hemoglobin method. Microhematocrit method was used in measuring the hematocrit described by Svobodova et al. (1991) and RBC and WBC was determined with a Burker hemocytometer using Natt and Herrick (1952) stain.

Red blood cell indices were calculated in accordance with Greer *et al.* (2014).

$$\textbf{MCV} = \frac{\text{Hematocrit}}{\text{RBC}}$$

$$\textbf{MCH} = \frac{\text{HGB}}{\text{RBC}}$$

$$\textbf{MCHC} = \frac{\text{HGB}}{\text{Hematocrit}}$$

Statistical Analysis

After finding possible results, information of growth, muscle composition and hematology were subjected to statistical analysis ANOVA and ±SE values was evaluated by using SPSS (Steel *et al.* 1996)and difference between means ($P < 0.05$) were evaluated by the Tukey's HSD Test (Snedecor & Cochran, 1991).

3. RESULTS AND DISCUSSION

Growth performance

Results revealed that Iron NPs and Iron oxide NPs had pronounced effect on the growth performance and muscle composition of *Labeo rohita* fingerlings. After the period of 90 days, body weight, body length and SGR were recorded highest in Fe-NPs and FeO_3 NPs treated groups. Moreover, highest body weight gain, body length and SGR was recorded in group T_2treated with FeO_3 NPs 0.8 mg/Kg (Table 2).

Body composition

Body composition parameters are the key indicator of the physiological status of fish as well as feed composition

and feeding rates. The results revealed various levels of Fe-NPs and FeO_3 NPs in diet showed pronounced effect on the muscle composition protein, lipid, ash and dry matter of experimental fish *Labeo rohita.* Crude lipid and crude proteins content were found highest in group T3 treated with Fe-NPs 0.6mg/Kg whereas ash contents and gross energy were recorded highest in group T4 treated with Fe-NPs 0.8mg/Kg. Moisture contents were recorded highest in group T1 treated with FeO_3 NPs 0.6mg/Kg and dry matter recorded lowest as well (Table 3).

Hematology

All the hematological parameters were lower significantly (P<0.05) in fish fed on control diet compared to those fed on experimental diets. Higher hemoglobin (Hb) contents were observed in fish fed with Fe NPs supplemented diet compared to those fed with Fe_2O_3 NPs which was 7.42 g/dl and red blood cell (RBC) count was significantly (P< 0.05) lower ($1.54x10^3$ /µL) in fish fed the Fe_2O_3 NPs supplemented diet than in those fed with Fe NPs supplemented diet while all of the red blood cell (RBC) indices MCV, MCH and MCHC were higher in fish fed with Fe_2O_3 NPs supplemented diet than in those fed with Fe NPs supplemented diet which were160.98 Fl, 58.8pg and48.58 g/dl, respectively. In comparison to fish fed with Fe NPs supplemented diet, white blood cell (WBC) count was significantly (P< 0.05) lower ($9.55x10^3$ /µL) in fish fed with the Fe_2O_3 NPs supplemented diet. In case of hematocrit (PVC%), it was maximum (23.97%) in fish fed with Fe NPs supplemented diet compared to those fed with Fe_2O_3 NPs supplemented diet (Table 4).

Discussion

Growth in fish is considered in terms of increase in volume and the volume is represented by weight. Results indicated that body weight, body length and SGR were observed highest in group T2 treated with FeO_3 NPs 0.8mg/Kg. These results are supported by Behera *et al.,* (2014) who found the significant (P<0.05) changes in growth parameters FW, WG, %WG, SGR and CF with the inclusion of Fe_2O_3-NPs. Small size of nano iron have facilitated the passage of nanoparticles across tissues and cell membrane leading to improved gastrointestinal absorption because due to much smaller size nano Fe can penetrate the cells easily and is more efficient in action thus leading to increase of body cells of the experimental fish and its growth increased. Protein content deposition in *Labeo rohita* body meat was recorded highest in group T3 treated with Fe-NPs 0.6mg/kg as compared to control group-T_0 and Fe_2O_3 NPs treated groups after the period of 90 days. These results are in agreement with the findings of El-Shenawy *et al.,* (2019) who found significantly high protein retention in the muscle of Nile Tilapia (*Oreochromis niloticus*) by administrating Fe-NPs at various levels in diet.

The reason is the adsorption of proteins occurs on the surface of nanoparticles. Nano-protein coronas can interfere with protein folding and can enhance protein cross linking and fibrillation thus lead to high protein retention in the body meat of experimental fish. Crude lipid deposition in *Labeo rohita* body meat was recorded highest in group T3 treated with Fe-NPs@0.6mg/kg as compared to control group-T_0 and Fe_2O_3 NPs treated groups which is in agreement with Akter *et al.,* (2018) who found significantly high lipid retention in the muscles of Bagridae catfish (*Clarias batrachus*) by administrating Fe-NPs at various levels in fish feed. The reason is Iron NPs bind lipoprotein in the blood formed complex resulted in high lipid metabolism high retention of lipid in the muscles of *Labeo rohita.* Highest ash contents were recorded in group T4 treated with Fe-NPs@0.8mg/Kg which is in agreement with the findings of two researchers El-Shenawy *et al.,* (2019) and Akter *et al.,* (2018) who stated that ash contents increased in Nile Tilapia (*Oreochromis niloticus*) and Bagridae catfish (*Clarias batrachus*) with the increasing level of Fe-NPs and high ash value observed in group treated with high concentration of Fe-NPs as compared to control group-T_0 and Fe_2O_3 NPs treated groups. Highest ash contents in fish treated with Iron-NPs are may be due to more bioavailability and more intake of iron NPs. Gross energy also recoded highest in group T4 treated with Fe-NPs@0.8mg/Kg because ash contents were highest in this group. Moisture contents recorded highest in group T1 treated with Fe_2O_3 NPs@0.6mg/Kg which may be due to higher surface area to volume ratio of NPs and dry matter is vice versa (Behera *et al.,* 2014).

Hematological parameters are one of the significant indicators, for observing health condition of fish in terms of physiological and pathological approach. A higher red blood cell (RBC) count and RBCs indices (MCV, MCH, MCHC) was observed in fish fed the nanoparticles supplemented diet compared to those fed control diet, which may be due to more iron intake in nano forms because at the nano-size dietary minerals can pass into the cells more freely than their larger complements and iron

is a major co-factor in blood formation in common and a main component of all RBCs in particular. It can be said that fish may experience gill injury because of iron toxicity. This may cause oxygen deficiency, causing more production of RBCs to lessen the effect. Gill injury induced by nanoparticles have reported by Ates *et al.* (2013) and Li *et al.* (2009) in gold fish and medaka fish, while similar results were found by Behera *et al.* (2014) who reported that RBCs count was significantly higher in Fe NPs- treated groups as compared to the control group. Similar results were shown by Karthikeyen *et al.* (2013) who have reported higher MCV, MCH and lower MCHC values in groups fed with Iron oxide nanoparticle compared to the control group. While Remya *et al.* (2015) reported in results that MCV, MCH and MCHC were decreased in group of fish fed with Iron oxide nanoparticles compared to the control group.

During the study period, WBCs count of fish blood were found positively correlated with dietary nanoparticles supplementation and expressively higher WBCs were observed for the fish group fed on the feed with 0.6mg/kg Iron nanoparticle. Increased numbers of WBCs might be linked with immunological reaction to produce antibodies to handle with stress induced by the nanoparticles. Uzo *et al.* (2018) demonstrated in results that fish fed with diet containing Iron oxide nanoparticles showed a significantly ($P< 0.05$) higher white blood cell (WBC) count compared to the control group.

4. CONCLUSION

The results revealed that both iron and iron oxide nanoparticles improved the growth, muscle composition and hematology of *Labeo rohita* as compared to the control diet. Some parameters were improved in the iron nanoparticles supplemented diet compared to the iron oxide nanoparticles supplemented diet and vice versa. Lower doses of the nanoparticles may provide good results so it is suggested that further research in this matter should be in that direction.

Highlights

- Low production of aquaculture is primarily due to improper diet (leads to retard growth).
- Diet supplemented with iron and iron oxide nanoparticle show improved health of *Labeo rohita* in terms of hematology and body composition.
- Iron oxide nanoparticle holds the abilities to enhance growth of *Labeo rohita.*

5. CONFLICT OF INTERESTS

All authors have declared that the there is no conflict of interest regarding this publication.

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Table 1. Composition of Control and Experimental Diets

Ingredients (g)	**Control diet**	**T_1 Iron Oxide NPs (0.6mg/kg)**	**T_2 Iron Oxide NPs (0.8mg/kg)**	**T_3 Iron NPs (0.6mg/kg)**	**T_4 Iron NPs (0.8mg/kg)**
Fish meal	25	25	25	25	25
Sunflower meal	18	18	18	18	18
Gluten	50	50	50	50	50
Wheat bran	1.7	1.7	1.7	1.7	1.7
Rice bran	1.7	1.7	1.7	1.7	1.7
Vitamin premix	1.3	1.3	1.3	1.3	1.3
Canola oil	2.3	2.3	2.3	2.3	2.3
Total	100	100	100	100	100
Iron oxide NPs (<50nm)	0	0.6	0.8	0	0
Iron NPs(<50nm)	0	0	0	0.6	0.8

Table 2. Comparison of means of Growth performance of *Labeo rohita* cultured under control and different treatments

Parameters	**T_0 (Control)**	**T_1**	**T_2**	**T_3**	**T_4**
	NPs (0.0mg/kg)	Iron Oxide NPs (0.6mg/kg)	Iron Oxide NPs (0.8mg/kg)	Iron NPs (0.6mg/kg)	Iron NPs (0.8mg/kg)
Body weight	4.97±0.36B	5.40±0.44B	5.59±0.46A	5.21±0.4A	5.25±0.41A
Body length	7.39±0.28A	7.21±0.26B	7.41±0.29A	7.23±0.2B	7.30±0.29AB
SGR	0. 985 ± 0.055A	1.100 ± 0.0A	1. 130 ± 0.010A	1.115±0.01A	1.085 ±0.045 A

Table 3. Comparison of means of approximate muscle composition of *Labeo rohita* cultured under control and different treatments

Parameters	**T_0 (Control)**	**T_1**	**T_2**	**T_3**	**T_4**
	NPs (0.0mg/kg)	**Iron Oxide NPs (0.6 mg/kg)**	**Iron Oxide NPs (0.8mg/kg)**	**Iron NPs (0.6mg/kg)**	**Iron NPs (0.8mg/kg)**
Moisture (%)	2.000 ± 0.000B	7.0 ± 0.00 A	2.50± 0.50 B	6.500 ± 0.50A	3.00±0.000B
Dry matter (%)	98.0 ±0.0A	93.0 ± 0.00 B	97.50± 0.50 A	93.500±0.50B	97.000 ±0.0A
Ash (%)	78.0±1.00B	87.0 ± 0.00 A	81.0± 1.0 B	85.500 ±0.5 A	88.50±0.50A
Crude lipid (%)	21.500 ± 1.50B	25.50±0.50AB	29.0± 0.0AB	33.000 ± 0.0A	32.5± 0.50 A
Crude protein (%)	25.0 ± 0.00 B	8.590 ± 0.78B	10.930±0.0 B	28.12 ±0.0A	27.3±0.78AB
GE(Kcal/g)	555.62± 7.99 B	531.28± 3.85B	572.02±2.05A	603.18±2.05A	613.71 ±3.8A

Table 4. Comparison of means of approximate hematological analysis of *Labeo rohita* cultured under control and different treatments

Parameters	**T_0 (Control)**	**T_1**	**T_2**	**T_3**	**T_4**
	NPs (0.0mg/kg)	Iron Oxide NPs (0.6mg/kg)	Iron Oxide NPs (0.8mg/kg)	Iron NPs (0.6mg/kg)	Iron NPs (0.8mg/kg)
Hemoglobin (g/dl)	6.67± 0.010 A	7.33± 0.215 B	7.03± 0.115 B	7.42± 0.130 B	7.40± 0.215 B
WBCs ($x10^3$ / µL)	7.17± 0.020 B	11.05± 0.050 A	9.55±0.020 A	12.235± 0.565 A	11.295± 1.065 A
RBCs ($x10^6$ /µL)	1.33± 0.020 A	1.65± 0.050 B	1.54± 0.135 B	8.615± 0.185 C	8.565± 0.135 C
HCT (PVC)%	20± 1.000 A	21.82± 0.410 B	18.3± 0.4 B	23.97± 0.135 B	21.77± 1.065 B
MCV (fL)	107.3± 0.000 D	160.98± 10.72 A	150.5± 8.270 B	122.47± 6.435 C	114.89± 13.165 CD
MCHC (pg)	34.17± 0.000 D	52.47± 2.500 B	58.8± 4.230 A	40.47± 0.035 C	41.80± 0.900 C
MCH (g/dl)	30.73± 0.000 C	48.58± 3.035 A	44.315± 0.850A	41.295± 1.235 B	39.74± 0.885 BC