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Electron beam irradiation on aqua-feed: Effect on haemato-immunological parameters and histological changes in *Labeo rohita* fingerlings

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ABSTRACT

A feeding trial for 60 days was conducted to study the effect of electron beam (EB) irradiated feed (10 KGy radiation) on the haemato-immunological parameters and histological changes in Labeo rohita (L. rohita) fingerlings. One hundred and eight fishes (5 ±0.5 g) were randomly distributed into 3 treatment groups with each of three replicates. The feed ingredients irradiated were cotton seed cake, rubber seed cake, soybean meal, mustard oil cake and rice bran. Three practical diets with 30% protein content were prepared viz control (non-irradiated diet), T1 (formulated feed treated with 10 KGy EB radiation) and T2 (feed formulated by individually irradiated ingredients). Haemoglobin content in the treatment groups was increased significantly but RBC count was similar (P>0.05) among the experimental groups. Immunological parameters like respiratory burst activity and total leucocytes count increased significantly in EB irradiated groups. Feeding EB irradiated diet affect the histological structure of the gill and liver of the fishes in different groups. The study concludes that EB irradiated diets improve the haemato-immunological parameters in L. rohita fingerlings.

Key words: Electron beam irradiation, respiratory burst activity, *Labeo rohita*, histology, WBC

INTRODUCTION

The projected global aquaculture production by 2021 is 79 million tons [1] considering the current growth rate. The average global animal feed production is 954 million tons; out of which aquaculture feed contribute to 34 million tons [28]. To reach the projected target, aqua-feed industry required almost three fold increase of aqua-feed by 2021. The requirements for aquaculture feed are likely to be further increased by an increasing trend towards the intensification of farmed production of carp species in Asian countries.

Fish meal, the conventional protein source is not cost effective and not available adequately to fulfil the demand. According to Rumsey [23] and Tacon [24] cost effective practical aqua feeds can be produced from ingredients other than fish meal without affecting the growth. Extensive research is carried out to replace the fish meal with some cost effective plant ingredients in aqua-feed. Various oil seed cakes and meals are produced on large scale as by product of the edible oil industry. These include soybean meal, cotton seed cake, ground nut oil cake, mustard oil cake etc. These plant protein sources are considered most viable alternative to replace fish meal for economic fish production in most developing countries. But the major problem associated with the use of plant based ingredients in fish feed is the presence of some anti-nutritional factors [22].

Anti-nutritional factors like phytic acid, tannin form complexes with proteins and digestive enzymes like proteases and amylases of the intestinal tract, thus inhibiting the digestibility [2]. Dietary tannin makes complex with iron and making it unavailable for haemoglobin production [3]. Several attempts have been made to incorporate oilseed based ingredients in aqua-feed through deactivation or partial removal of these anti-nutritional factors. Many traditional methods such as thermal processing, soaking, addition of exogenous enzyme, application of dry heat etc are available for reducing the anti-nutritional factors [4, 5]. Use of these techniques found to be less effective, labour intensive and will lead to the loss of vital nutrients. Although, gamma irradiation has wide applications, the presence of radioactive residues in feed is a limiting factor. This has opened up the possibilities of employing the EB irradiation as a safe and reliable physical method of irradiation. Free- electron is one of the newest dimensions of irradiation of agricultural products. The high- energy electron beam which once passed through materials brings about physical, chemical and biological changes. EB irradiation at a dose of 10 KGy has been found to be effective to reduce the antinutritional factors present in plant based unconventional feed ingredients [6]. Decontamination of food and agricultural commodities by EB irradiation has been proved to be successful without any apparent detrimental effects [25, 26, 27].

L. rohita being the important species extensively cultured in India, contributing 84% of the total aquaculture production. The protein requirement of *L .rohita* is 30% [30] and has huge potential for utilization of plant based ingredients in the carp feed. But prior to the commercialisation of the technology, basic information regarding its safe concentrations, effect upon dietary nutrients, effect on fish physiology need to be investigated. With this background, the present study is designed to investigate the haemato-immunological parameters and histological changes of *L. rohita* fingerlings fed with EB irradiated feed.

MATERIALS AND METHODS

Irradiation of feed samples and experimental diets preparation

Experimental diets were formulated with 30% protein and the feed formulation is given in Table 1. All the ingredients were ground, weighted as per the formulation and mixed well to form dough with the addition of necessary quantity of water. When the dough was formed, the required amount of the oil was incorporated in it, mixed well and placed in the cooker for 30 min. After cooking, vitamin and mineral premix was added upon cooling. Dough was pelletized and air dried (1-2 hrs) and kept in hot air (50^0C) over night for complete drying. After drying, pellets were packed in two polythene bags, one bag was given 10 KGy EB irradiation (T1 diets), while other bag was not given any irradiation (Control diets). Mean while, feed ingredients such as soybean meal, cotton seed cake, rubber seed cake, mustard oil cake and rice bran were irradiated individually with 10 KGy EB radiation and the T2 diet was formulated using these irradiated ingredients following the same procedure.

Table 1. Composition of the experimental diets fed to *L. rohita* fingerlings

Ingredients	Percentage (%)
Soybean meal	25.0
Cotton seed cake	20.0
Rubber seed cake	20.0
Mustard oil cake	20.0
Rice bran	5.0
Cod liver oil	6.0
Vitamin and mineral mixture	2.0
CMC	2.0
BHT	0.02

Composition of vitamin mineral mix (Agrmin) (quantity/kg)

Vitamin A-6,25,000 IU; Vitamin D_3-62,500 IU; Vitamin E-250mg; Nicotinamide-1g; Cu-312mg; Co-45mg; Mg-6g; Fe-1.5g; Zn-2.13g; I-156mg; Se-10mg; Mn-1.2g; Ca-247.34g; P-114.68g; S-12.2g; Na- 5.8mg; K-48.05mg.

Experimental design

The experimental set up consisted of six plastic rectangular tanks (57 X 36 X 47 cm, 75 L capacity). One hundred and eight (108) fingerlings (5±0.5g) of *L. rohita* were randomly distributed in 3 distinct experimental groups viz. control, T1 and T2 in triplicate. Among these, T1 indicates the groups fed with formulated diets after irradiation and T2 indicates the group fed with diet made up of individually irradiated ingredients.

Feeding trial

The fishes were fed to satiation with different experimental diets for a period of 60 days. Feeding was adjusted to the biomass after every sampling at 10 days interval and the daily ration was divided into 2 equal parts and fed.

Physiochemical parameters of water

Water quality parameters were maintained throughout the culture period within the normal range viz temperature (24-28°C), pH (7.2-7.5), dissolved Oxygen (6-7.5 ppm), free Carbon dioxide (0 ppm), total alkalinity (180-210 ppm), total hardness (180-195 ppm) and ammonia (0.00- 0.05ppm).

Haemato-Immunological parameters

Collection of blood

Four fishes from each experimental group were anesthetized using clove oil and the blood was withdrawn from vena caudalis using a medical syringe and transferred immediately to test tube containing thin layer of EDTA powder and mixed well in order to prevent haemolysis.

Haemoglobin percentage

The haemoglobin content of blood was analyzed following the cyanmethemoglobin method using Drabkins Fluid (Qualigens). Blood (20 µl) was mixed with 5 ml of Drabkins working solution in a test tube and the absorbance was measured at 540 nm wave length. The final concentration was calculated by comparing with the standard cyanmethemoglobin (Qualigens) by using the following formula:-

haemoglobin (g/dL)= [OD(T)/OD(S)]×[251/1000]×60,

where OD(T) is the absorbance of the test, OD(S) is the absorbance of the standard.

Total RBC and WBC counts

Total RBC and WBC were counted using the RBC and WBC diluting fluids (Qualigens), respectively. Blood (20µL) was mixed with corresponding diluting fluid (3980 µL) in a clean test tube and was shaken well to suspend the cells uniformly in the solution. The cells were counted in a haemocytometer using high power (40X) of light microscope.

The following formula is used to calculate the number of RBC and WBC

No. of cells (mm^{-3}) = No of cells counted X dilution / Area counted X depth of fluid

Respiratory burst activity

Nitro blue tetrazolium (NBT) assay was carried out as of Secombes [29] and as modified by Stasiack and Baumann [7]. Blood (50 µl) was placed into the wells of 'U' bottom microtitre plates and incubated at 37^0C for 1hr to facilitate adhesion of cells. Supernatant was added with 0.2% NaCl (50µl) after PBS washing (3 times) and was incubated for 1hr. The cells were then fixed with 100% methanol (2-3 minutes) and again washed thrice with methanol (30%) and then air dried. Each well was added with 2N potassium hydroxide (60 µl) and dimethyl sulphoxide (70 µl). The OD of the turquoise blue solution was then read in ELISA reader at 540 nm.

Serum protein, albumin and globulin

Serum protein was estimated by biuret method [8] using the kit (Colagen). Biuret reagent (1 µl) was added with 50 ml of serum, mixed well and incubated at 37^0C (10 min) and the absorbance was measured at 630 nm. Albumin was estimated by the bromocresol green binding method [9]. The absorbance of the standard and test was measured at 630 nm. Serum globulin was determined by subtracting serum albumin from total protein. The albumin globulin ratio (A/G) was determined.

Histological analysis

Fish gill and liver were collected and preserved in 10% neutral buffered formalin for 72 hrs. Samples were further processed as per the routine laboratory protocol and sections of 5µ size were stained with haematoxylin and eosin stains. Slides were observed under Zeiss Digital Microscope using Axiovision software.

Statistical analysis

The mean values were analyzed by using the statistical software SPSS version 14.0. Mean values between treatments were compared using Duncan multiple range test. Difference were considered at 95% level of significance ($P<0.05$).

RESULTS

The blood hemoglobin, total WBC counts and RBC count of the *L. rohita* fingerlings of different treatment groups are shown in Table 2. Both the irradiated diet fed groups showed significant increase in hemoglobin content compared to control group. Among hematological parameters analyzed, both T1 and T2 groups did not recorded significant difference between each other except for WBC count.

The production of superoxide examined by NBT reduction in *L. rohita* fingerlings was significantly ($P<0.01$) influenced by irradiation (Table 2). Both the irradiated diet fed groups showed significant increase in NBT compared to the control group, where as the NBT value between T1 and T2 groups showed no significant difference from each other.

Table 2: Total WBC ($10^{5/}$mm), RBC ($10^{6/}$mm), Haemoglobin (gm %) and NBT (A620) level in *L. rohita* fingerlings fed with EB irradiated diet at the end of experiment

	WBC	RBC	Haemoglobin content	NBT
Control	1.58^{c} ±0.34	1.94^{a}±.05	8.6^{b}±0.20	0.24^{c} ±0.004
T1	2.96^{a}±0.89	1.95^{a} ±0.08	14.24^{a}±0.24	0.40^{ab}±0.001
T2	2.46^{b}±0.5	1.92^{a} ±0.1	13.98^{a} ±0.14	0.41^{a} ±0.001

Mean value in the column with different superscript differ significantly (P<0.05). Data expressed as mean ±SE. Values recorded in percentage on wet weight basic were arcsine transformed for testing the variance.

Total protein, albumin, globulin values were significantly higher in the irradiated groups than the control group (Table 3). But T1 and T2 groups were similar for total protein. Albumin globulin ratio A/G ratio did not vary significantly among all the experimental groups.

Table 3: Total protein, albumin, globulin (gm %) and A/G ratio in *L. rohita* fingerlings fed with EB irradiated diet at the end of experiment

	Total Protein	Albumin	Globulin	A/G ratio
Control	4.37^{b} ±0.01	1.09^{b} ±0.001	3.28^{c} ±0.10	0.33^{a} ±0.04
T1	6.23^{a} ±0.001	1.53^{a} ±0.02	4.70^{a} ±0.12	0.33^{a} ±0.002
T2	7.02^{a} ±0.03	1.50^{a} ±0.001	4.54^{b} ±0.01	0.33^{a} ±0.01

Mean value in the column with different superscript differ significantly (P<0.05). Data expressed as mean ±SE. Values recorded in percentage on wet weight basic were arcsine transformed for testing the variance. A/G = albumin: globulin

Gill tissue from control fish exhibited swollen primary lamellae and extensive hyperplasia of secondary lamellae along with severely damaged secondary epithelial cells and complete loss of secondary lamellae at some places (Fig 1). Whereas gill tissue from both the treatment fish T1 and T2 showed marked improvement in the secondary lamellae and secondary epithelial cells showed only mild basal hyperplasia (Fig. 2 and 3). Liver tissue from control group exhibited multiple necrotic focal areas occupied with atrophied acinar cells. Liver parenchyma also showed extremely damaged hepatic cords and atrophied hepatocytes (Fig.4). Whereas sections of liver tissue from treatment fish showed marked improvement in the liver parenchyma, only few mild focal necrosis could be noticed. Well arranged pancreatic acinar cells were seen between the hepatocytes in both the treatment groups (Fig. 5 and 6).

Fig 1. Gill tissue of *L. rohita* fingerlings fed control diet showing swollen primary lamellae and hyperplasia of secondary lamellae along with severely damaged secondary epithelial cells

Fig 2. Gill tissue of *L. rohita* fingerlings fed with T1 diet showing no visible change

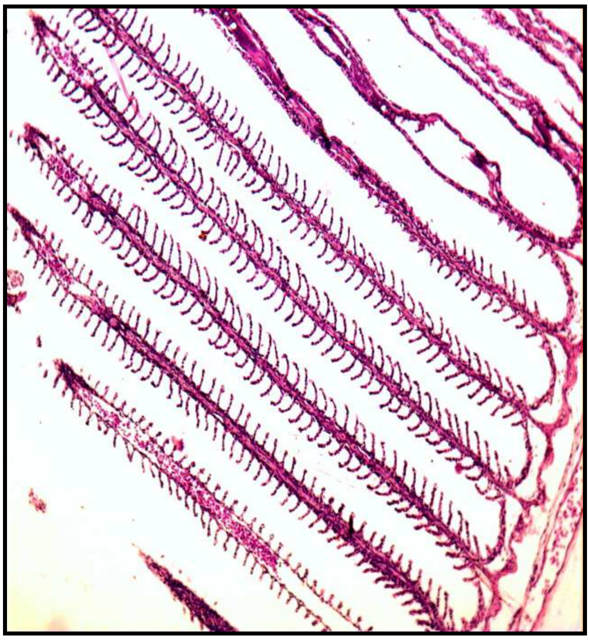

Fig 3. Gill tissue of *L. rohita* fed with T2 diet showing no visible changes

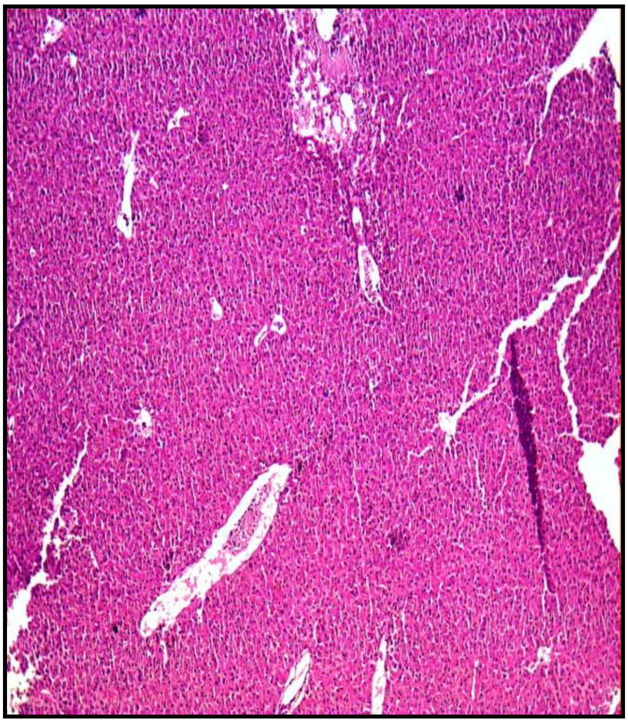

Fig 4. Liver tissue of *L. rohita* fingerlings fed with control diet exhibited multiple necrotic focal areas occupied with atrophied acinar cells

Fig 5. Liver tissue of *L. rohita* fingerlings fed with T1 diets exhibited normal with few mild focal necrosis

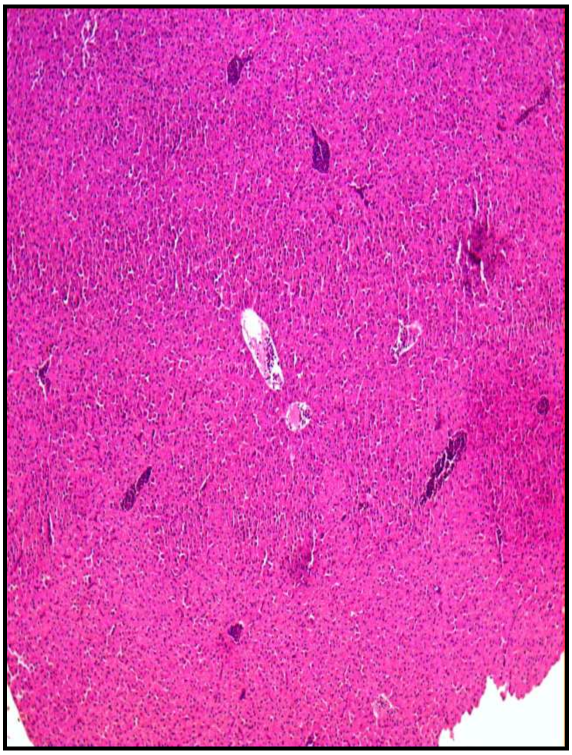

Fig 6. Liver tissue of *L. rohita* fingerlings fed with T2 diet showed normal with mild focal necrosis

DISCUSSION

The haemoglobin content of *L. rohita* fingerlings reduced significantly in non-irradiated diet fed groups as compared to irradiated diet fed groups. The decrease of haemoglobin in non-irradiated diets may be due to binding of tannins with Fe^{2+} ions, which forms the central core of the haemoglobin molecule, making it unavailable as evidenced by Afsana et al. [3]. But Mmereole et al [13] reported that boiler chicken, fed with non-irradiated rubber seed cake diet did not affect the haemoglobin content.

No significant variation in RBC count among different treatment and control groups were recorded by the present study. However, both the irradiated diet fed groups showed significantly higher levels of WBC count than the non-irradiated diet fed groups. Mmereole et al [13] reported reduction in WBC count in broiler chicken, fed with rubber seed cake based diets. WBC is an important component of immunoglobulin for antibody production and reduction of WBC count may leads to reduction in immunity. In the present study, WBC were reduced significantly in non-irradiated diets, indicated that feeding of raw plant based diets having higher level of anti-nutritional factors may be detrimental to fish by suppressing its immuno-stimulatory effect.

Fish phagocytes are able to generate superoxide ions (0_2^-) and its reactive derivatives (i.c hydrogen peroxide and free radicals) during a period of intense oxygen consumption called the respiratory burst activity [21,17]. These reactive oxygen derivatives are considered to be toxic for fish bacterial pathogens [17, 14] and are generated by phagocytes upon stimulation. The stimulation of the phagocytic cell membrane leads to increase consumption of oxygen, the reduction of which is catalyzed by a membrane bound enzyme NAD(P)H oxidase which gives rise to 0_2^- [15]. So, it is evident that increased respiratory burst activity can be correlated with increased bactericidal activity of phagocytes [16], hence a better immunity. In the present study the NBT value was significantly higher in irradiated diet fed group than the non irradiated diets fed group. EB irradiation at 10 KGy can effectively reduce the antinutritional factors in soybean meal, cotton seed cake [6]. Hence, the higher respiratory burst activity in the irradiated diet fed group may be attributed to the reduction of anti-nutritional factors by the EB irradiation and thereby, reduces the stress, leading to higher immunity.

Proteins are the most important compounds in the serum with albumin and globulin being the major serum proteins. The gamma globulin fraction is the source of almost all the immunological active protein of the blood and are absolutely essential for maintaining a healthy immune system. Increase in the serum protein, albumin and globulin levels are considered a stronger innate response in fishes [18]. In the present study, serum protein, albumin and globulin were significantly higher in irradiated diet fed group than the non-irradiated diet fed group. Higher values of serum protein and globulin in irradiated diet fed group indicates that EB irradiated diet gives a healthy immunity system in *L. rohita* fingerling than the non-irraadiated diet. Mmereole et al [13] reported the reduction in serum protein level of broiler chicken, fed with raw rubber seed cake based diets. But, El-Neily [12] reported feeding rats on gamma-irradiated cotton seed meal at 10 KGy caused no significant changes of total protein and albumin concentration. In the present study the albumin-globulin ratio of the experimental groups did not show any significance different indicating, no immuno-suppressive effect of EB irradiated diets on *L. rohita* fingerlings.

The gill tissue of *L. rohita* fingerlings fed with non irradiated diets showed swollen primary lamellae and extensive hyperplasia of secondary lamellae along with severely damaged secondary epithelial cells and loss of secondary lamellae at some places. However, none of these gill pathology were evident in the fishes of both the treatments groups fed with irradiated diets. This is substantiated by the finding of Roman & George [19] who reported hyperplasia and swollen in primary lamella in *onchorhynchus mykiss* exposed to high dietary tannin. Varanka et al [20] reported marked necrosis in the liver and redness of tissue when exposed to tannic acid in common carp. In the presence study the liver tissue from control fishes showed multiple necrotic focal areas occupied with atrophied acinar cells which may be due to toxic effect of tannin in the nonirradieted diet fed groups.

CONCLUSION

From this study, it can be concluded that haematological parameters of *L. rohita* fingerlings were affected except for RBC due to feeding of EB irradiated diet. Immunological parameters of the *L. rohita* fingerling fed with EB irradiated diet were also stimulated as evident by increased total leucocytes count and increased respiratory burst activity. This indicates that EB irradiated diet has immune-stimulating effect in *L. rohita* fingerlings.

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