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Effect of gamma irradiation on inactivation of *Ichthyophthirius multifiliis* trophonts and its efficacy on host response in experimentally immunized rainbow trout (*Oncorhynchus mykiss*)

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Abstract: The present study was conducted to investigate the effects of gamma irradiation on *Ichthyophthirius multifiliis* trophonts and to determine the effects of exposure on inactivated parasites in rainbow trout. In the first trial, fish were treated with irradiated *Ichthyophthirius multifiliis* trophonts for 21 days. Results showed that irradiation at doses of 150 Gy and higher was required to inactivate *Ichthyophthirius multifiliis* trophonts. The LD50 value obtained was 52.2 Gy. In the second trial, the effects of irradiated *Ichthyophthirius multifiliis* trophonts on physiological and biochemical components of blood and mucus, as well as skin morphology, were studied for 30 days. Results showed that total protein levels increased in fish exposed to 100 and 150 Gy irradiated trophonts. Fish exposed to 150 Gy treated parasites showed significant increases in serum lysozyme activity. Serum alkaline phosphatase levels were significantly lower in the treatment groups. In the skins of fish exposed to 100 and 150 Gy irradiated trophonts, a higher percentage of goblet cells was observed. Mucus protease and esterase levels were also higher in the treatment groups compared to the control group on days 20 and 30. This suggests that rainbow trout appear to benefit from treatment with gamma-irradiated *Ichthyophthirius multifiliis* trophonts, especially at 150 Gy.

Key words: Gamma irradiation, *Ichthyophthirius multifiliis*, trophonts, rainbow trout

1. Introduction

The freshwater ciliate *Ichthyophthirius multifiliis* (*I. multifiliis*), one of the most important protozoan pathogens of farmed and wild fish populations, causes significant economic losses to aquaculture industries worldwide (1). The 3 stages in the life cycle of the parasite are an infective theront, a parasitic trophont, and a reproductive tomont (2). It is difficult to control this parasite using chemotherapy after penetration into fish skin and gills. The high cost involved in therapy and the public concern for food and environmental safety are other disadvantages of chemotherapy. Thus, vaccination against *I. multifiliis* can be considered an alternative to chemical treatments to prevent mortality (2) in fish. A number of studies have indicated that whole-cell trophonts, theronts, and ciliary protein preparations or recombinant i-antigen proteins of this parasite are able to induce an acquired immune response in numerous fish species, including the channel catfish (*Ictalurus punctatus*), trout (*Salmo gairdneri*), and carp (*Cyprinus carpio*) (2–5). Trophonts are commonly inactivated by using 1%–4% formalin (5) or freezing (5) and used for immunization at different doses (5).

Gamma radiation is widely used by many researchers to inactivate parasites for the preparation of vaccines, instead of traditional thermal or chemical methods of inactivation. This technology appears to create a vaccine that is more effective than so-called "killed" vaccines against disease, and has the added advantage of a longer storage life than "live" vaccines. Irradiation is a technically simple process that retains the structural features of the microbial pathogen without destroying the natural antigens or the intrinsic adjuvant. Therefore, a strong immune response

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is induced in the vaccinated host (6). Inactivation and weakened infectivity of protozoan and helminth parasites, bacteria, and viruses by gamma radiation have been studied previously (6–9).

There are no available data on the effectiveness of gamma irradiation in producing an antiparasite radiovaccine in fish species. Therefore, the present study was designed preliminarily to determine firstly the optimum dose of gamma irradiation to produce inactivated trophonts of *I. multifiliis* and secondly its effect on host response in experimentally immunized rainbow trout, especially in terms of their serum enzyme, serum protein, mucus components, and skin morphology.

2. Materials and methods

2.1. Fish

Healthy rainbow trout weighing 30–40 g prepared from a fish farm in Karaj, Iran were kept in running water (flow rate 0.4 L/s) in polypropylene tanks (300 L) with water temperature 15 ± 1 °C, dissolved oxygen 5.2 ppm, and natural photoperiod (10 L:14 D).

2.2. Parasite propagation

I. multifiliis was obtained from heavily infected rainbow trout caught from a local fish farm. Mature trophonts were collected and transferred to glass aquaria containing aerated tap water at 17–18 °C until all individuals were heavily infected by the ciliate parasite. After 72 h the trophonts in infected fish were collected and counted to prepare vials containing 100 trophonts in each vial.

2.3. Gamma irradiation

After the collection of trophonts, a Nordian model 220 gamma cell instrument with a dose rate of 4.8 Gy/s and activity 20,469 Ci was used for parasite inactivation. Different doses of gamma rays (50, 100, 150, 200, 250, and 300 Gy) were used for irradiation of parasite samples. For each dose, 3 vials of parasites were subjected to gamma irradiation. The irradiation process was performed on the parasite samples frozen in dry ice.

2.4. LD50 to determine the optimum dose for *I. multifiliis* inactivation

One hundred and eighty rainbow trout were distributed equally into 6 groups with 30 fish in each group. Each group contained 10 fish in triplicate reared in separate aquaria (12 L) with water temperature set at 17–18 °C. Each vial of gamma-irradiated or active trophonts was used for bathing fish in each aquarium. Twenty-one days after the challenge, all fish in each aquarium were collected and trophonts in each fish were counted.

2.5. Experimental design

Two hundred and forty fish were allocated in 4 groups (in triplicate) of 20 fish distributed per aquarium. One group was the control fish and other groups were fish exposed to gamma-irradiated *I. multifiliis* at 100, 150, and 250 Gy for 30 days. Similar to the LD50 test, each vial of gamma-irradiated or active trophonts was used for bathing fish in each aquarium. Doses were selected according to results achieved in the LD50 test.

2.6. Blood collection

On days 20 and 30, 7 fish from each aquarium were anesthetized with a clove oil bath (50 µL/L), and then bled from the caudal peduncle by heparin syringes. Serum was isolated by centrifugation (3000 × *g* for 5 min). Isolated serum was maintained at –20 °C for further experiments.

2.7. Lysozyme activity

Lysozyme activity was determined according to the method of Heidarieh et al. with slight modifications (10). Twenty-five microliters of serum samples were added to 75 µL of *Micrococcus lysodeikticus* suspension (75 µg/mL) prepared with 0.1 M phosphate citrate buffer, pH 5.8, in wells of a 96 well-plate in triplicate. The absorbance was measured continuously for 1 h at 450 nm. A unit of lysozyme activity was defined as the amount of a sample causing a decrease in absorbance of 0.001 per min and expressed as U/mg of the serum sample.

2.8. Assessment of alkaline phosphatase and total protein levels

The alkaline phosphatase level was estimated using the enzymatic colorimetric method of Rietman and Frankel (11). In this assay, the amount of pyruvate produced by forming 2,4-dinitrophenylhydrazine was measured at 546 nm. The result was expressed as U/mL of serum. Serum levels of total protein were determined according to the biuret method using an available commercial kit (Ziestchem diagnostics Co. Ltd., Tehran, Iran) on an autoanalyzer. The optical density was read at 630 nm and bovine serum albumin was used as standard.

2.9. Collection and preparation of skin mucus

Mucus collection was performed using the method of Sheikhzadeh et al. (12). On days 20 and 30 of the trial, 10 fish in each aquarium were anesthetized with clove oil (50 µl/L) and the skin mucus was collected by gentle scraping with enough care to avoid contamination with blood and homogenized with 4 volumes of Tris-buffered saline (TBS, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl). The homogenate was centrifuged at 4000 rpm for 30 min at 4 °C and the supernatant was lyophilized following freezing at –20 °C and –80 °C, respectively. After dissolving 300 mg of the lyophilized skin mucus powder in 1 mL of TBS buffer, centrifugation was performed at 4000 rpm for 30 min at 4 °C to separate the undissolved mucus portion. The supernatant obtained was aliquoted into 2 mL tubes, freeze dried, and stored at –20 °C.

2.10. Protease activity

Protease activity was determined using the azocasein hydrolysis assay according to the method of Palaksha et al. (13). Equal volume of mucus sample was incubated with 100 mM ammonium bicarbonate buffer containing 0.7% azocasein, pH 7.8, for 19 h on a shaker at 30 °C. The reaction was stopped by adding 4.6% trichloroacetic acid followed by a 10 min centrifugation at 10,000 rpm. Equal volume of the resultant supernatant was added to the microplate well containing 0.5 M NaOH and the increase in the OD values was measured at 405 nm.

2.11. Esterase activity

Esterase activity was determined according to the method of Sheikhzadeh et al. (12). Equal volumes of mucus samples were incubated with 0.4 mM para-nitrophenyl myristate in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100, pH 7.8 at 30 °C. The absorbance was measured continuously for 2 h at 405 nm by an ELISA reader. One unit of activity was defined as the amount of enzyme required to release 1 mmol of para-nitrophenyl product in 1 min.

2.12. Histological examination

Samples of skin from 5 fish were collected from each aquarium. They were fixed in 10% buffered formalin for 48 h and then processed following the histological methods. Briefly, the samples were dehydrated in alcohols and xylene, and then embedded in paraffin. Five-micrometer sections were obtained by microtome, rehydrated in alcohols, and stained with hematoxylin–eosin. The goblet cell (density per centimeter) in fish skin was then defined.

2.13. Statistical analysis

All the measurements were made in triplicate. The results were subjected to analysis of variance (ANOVA) followed by a least significant differences (Tukey) test. Correlation coefficients were significant with $P < 0.05$.

3. Results

Based on a dose/parasite proliferation curve, the control group exposed to active *I. multifiliis* trophonts had higher levels of infection than the treatment groups. Meanwhile, *I. multifiliis* trophont numbers on fish samples were decreased by increasing the gamma ray dose. From 150 Gy, parasite proliferation in fish samples stopped completely. The LD50 value obtained was 52.2 Gy (Figure).

Serum total protein levels increased significantly in fish exposed to 100 and 150 Gy gamma-irradiated *I. multifiliis* trophonts. The specific activity of lysozyme was higher in groups treated with 150 Gy gamma-irradiated parasites than the control group during days 20 and 30 of the trial. The serum alkaline phosphatase level was significantly lower in fish exposed to 100 and 250 Gy irradiated parasites on day 20, whereas on day 30 a significant decrease was shown in fish treated with 100 and 150 Gy of gamma-irradiated parasites (Table 1).

In fish skin, the epithelial goblet cells (density per centimeter) appeared to be higher in fish treated with 100

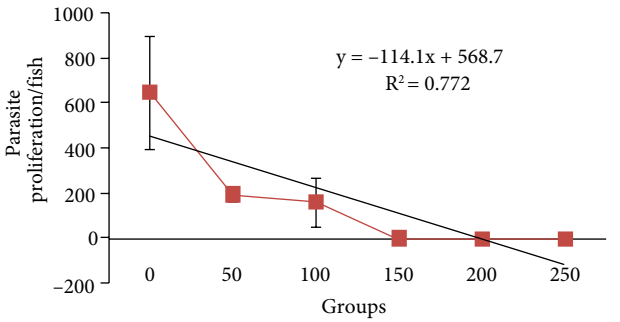

Figure. Dose per parasite proliferation curve for different doses of gamma-irradiated *Ichthyophthirius multifiliis* trophonts; Control group (1); 50 Gy (2); 100 Gy (3); 150 Gy (4); 200 Gy (5) and 250 Gy (6).

Table 1. Serum enzyme activity and immune parameters in rainbow trout treated with different doses of gamma-irradiated *Ichthyophthirius multifiliis* trophonts.

Treatments	Total protein (g/dL)		Lysozyme activity (U/mL)		Alkaline phosphatase (U/mL)	
	Day 20	Day 30	Day 20	Day 30	Day 20	Day 30
Control	2.03 ± 0.66 [a]	2.10 ± 0.33 [a]	26.71 ± 0.31 [a]	25.01 ± 0.01 [a]	125.33 ± 19.39 [a]	122.30 ± 12.05 [a]
100 Gy	3.39 ± 0.66 [b]	3.33 ± 0.26 [b]	30.03 ± 1.04 [a]	31.05 ± 2.02 [ab]	68.04 ± 30.7 [b]	48.55 ± 14.04 [c]
150 Gy	3.66 ± 0.54 [b]	3.41 ± 0.15 [b]	40.54 ± 0.59 [b]	38.95 ± 6.20 [b]	108.60 ± 17.05 [a]	78.31 ± 33.42 [b]
250 Gy	3.08 ± 0.44 [ab]	2.33 ± 0.11 [ab]	31.63 ± 2.67 [a]	28.72 ± 3.71 [a]	82.05 ± 22.14 [b]	116.30 ± 36.67 [ab]

The same superscript letters in the same column are not significantly different at $P < 0.05$.

and 150 Gy gamma-irradiated parasites during days 20 and 30 of the trial. The mucus protease level, determined by the azocasein hydrolysis assay, was significantly ($P < 0.05$) influenced by treatments with all doses of gamma-irradiated parasites. Similarly, the analysis of mucus esterase levels showed significant increases in all treatment groups during sampling on both days of the trial (Table 2).

4. Discussion

From this preliminary experiment it appeared that gamma irradiation at doses of 150 Gy and higher was required to inactivate *I. multifiliis* trophonts. Fish immunized with irradiated trophonts did not develop clinical symptoms of ichthyophthiriasis. It seems that the irradiated trophonts can be a good model for *I. multifiliis* vaccine development, because normal multiplication and infectivity of parasites can be suppressed by a small fraction of the dose required to kill the parasite. In this situation, the irradiation process retains the structural features of the pathogen without destroying the natural antigens or the intrinsic adjuvant (6). Therefore, a strong immune response is induced in the host.

With a high number of parasites per fish, serum alkaline phosphatase activity of the control group was higher than that of the treatment groups. The cause of this increase in alkaline phosphatase activity in the blood is unknown but may be related to high levels of stress due to the high number of parasites in the fish (14).

In protective mechanisms of fish against the *I. multifiliis* parasite, the role of specific antibodies has been emphasized as a result of the fact that both ELISA and immobilization titers increase following infection (15). However, a number of other factors could confer immunity against *I. multifiliis* infections. In this study, elevated lysozyme activity was observed in fish exposed to 150 Gy of irradiated trophonts. Similarly, in a previous study, following immunization of rainbow trout with live theronts higher lysozyme activity was shown (15). Meanwhile, in our study, serum protein levels increased in fish exposed to 100 and 150 Gy gamma-irradiated parasites. Blood protein includes the humoral elements of the nonspecific immune system such as immunoglobulin. It was suggested previously that higher lysozyme levels could be indicative that other nonspecific elements are activated by *I. multifiliis* infection (15). Higher protein levels in this study could prove the presence of some other nonspecific components in fish serum treated with gamma-irradiated parasites.

Fish epidermal mucus is the first line of defense against invading pathogens. The epidermal layer is also important in this aspect as it secretes the layers of mucus important to host defense mechanisms as well as providing mechanical protection (16). Proteases are considered to be involved in the regulatory production of antimicrobial peptides. Serine and cysteine proteases are involved in the defense against bacteria and protozoa by lysing the parasite (17). Cathepsin D and matrix metalloproteases are also involved in the production of antimicrobial peptide, parasin I, in the fish mucus (18). In this study, increased epidermal protease activity was observed in all treatment groups. In previous studies, increased epidermal protease activity was shown following the administration of some immunostimulants such as Ergosan and Hilyses in rainbow trout (12). Esterase is also thought to be important for protection against pathogen invasion (13). Esterase activity from treatment groups demonstrated a significant increase during this trial. Higher esterase activity secreted in the skin mucus of fish administered Ergosan and Hilyses was also previously shown (12). The mucus-producing cells in the fish epidermal and epithelial layer have been reported to vary with season, sex, species, nutrition, and environment, which could influence the mucus composition. An increase in the number of mucus-producing cells was observed in the skin of Atlantic halibut larvae following supplementation with *Lactobacillus plantarum* (19) and rainbow trout after treatment with

Table 2. Skin mucus components and goblet cell density in rainbow trout treated with different doses of gamma-irradiated *Ichthyophthirius multifiliis* trophonts.

Treatments	Skin epidermis goblet density/cm		Esterase (U/mg)		Protease (U/mg)	
	Day 20	Day 30	Day 20	Day 30	Day 20	Day 30
Control	746.60 ± 8.80 ^{a}	760.40 ± 5.70 ^{a}	15.40 ± 0.16 ^{a}	17.09 ± 0.20 ^{a}	11.80 ± 0.05 ^{a}	15.10 ± 0.10 ^{a}
100 Gy	940.10 ± 53.75 ^{b}	978.20 ± 42.23 ^{b}	32.51 ± 2.11 ^{b}	37.51 ± 4.40 ^{b}	28.65 ± 1.82 ^{b}	31.69 ± 3.73 ^{bc}
150 Gy	840.60 ± 36.74 ^{b}	1300.40 ± 120.40 ^{c}	28.68 ± 0.34 ^{b}	53.48 ± 10.90 ^{c}	40.30 ± 3.10 ^{c}	41.04 ± 2.90 ^{c}
250 Gy	621.66 ± 51.70 ^{a}	882.50 ± 44.98 ^{ab}	32.45 ± 3.06 ^{b}	59.51 ± 3.14 ^{c}	27.80 ± 1.79 ^{b}	38.12 ± 7.50 ^{bc}

The same superscript letters in the same column are not significantly different at $P < 0.05$.

Ergosan (20) and fermented *Saccharomyces cerevisiae* (21). It can be assumed that an enhanced immune system in the skin mucus of the fish treated with gamma-irradiated trophonts was partially due to increased mucous cell numbers and consequently accelerated release of mucous cell contents as a defense response.

In general, this preliminary investigation demonstrated that it is feasible to use gamma radiation technology to inactivate *I. multifiliis* trophonts. Meanwhile, rainbow trout appear to benefit from gamma-irradiated *Ichthyophthirius multifiliis* trophonts especially at 150 Gy in terms of enhanced physiological and biochemical components of blood and mucus. More studies on the minimum dose of radiation that adequately attenuates the parasite, optimum dose, and number of parasites for eliciting protective immunity are needed. For a comparison of efficacy with other available vaccines, further immunization trials on various fish host species are necessary to clarify the possibility of a gamma-irradiated *I. multifiliis* vaccine in fish species.

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