Int. J. Aquat. Biol. (2023) 11(5): 431-438
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Histopathological changes in the skin and gut mucus layers of rainbow trout (*Oncorhynchus mykiss*) challenged with *Ichthyophthirius multifiliis* inactivated by gamma rays and formalin

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Abstract: *Ichthyophthirius multifiliis*, a protozoan parasite, is a significant problem for fish farmers and Aquarium fish worldwide. This study aimed to evaluate the immunization of rainbow trout with gamma-irradiated, formalin inactive, and live theronts of *I. multifiliis*. In this study, fish were exposed to gamma-irradiated, formalin-inactivated, and live *I. multifiliis* theronts. Then, the histopathological changes in the mucous layers of the skin and intestines were studied after 7 and 14 days of exposure. Although no significant morphological changes were observed in the skin and intestines of the treated fish, the number of skin goblet cells increased significantly in fish treated with formalin-inactivated, gamma-inactivated, and live trophonts on 7 and 14 days. Compared to the negative control group, an increase in epidermal thickness on the skin was observed in fish challenged with formalin-inactivated, gamma-inactivated, and live trophonts. The numbers of mucous cells/total enterocytes in the intestinal epithelium of fish exposed to gamma-irradiated, formalin-inactivated, and trophonts live were higher than in non-infected fish. Moreover, a significant increase was found in the mucous cell numbers of the pyloric fold in treated fish with gamma-irradiated and formalin inactive trophonts at the first and second weeks. The results showed that the gamma-irradiated trophonts and formalin inactive trophonts could be safe for use in rainbow trout against *I. multifiliis*.

Article history:
Received 7 May 2023
Accepted 19 October 2023
Available online 25 October 2023

Keywords:
Rainbow trout
Parasite
Histopathology
Gamma-irradiated trophonts

Introduction

Ichthyophthirius multifiliis is a protozoan parasite that infects freshwater fishes' skin and gills, causing white spot disease (Wang et al., 2019). Studies show that the prevalence of white spot disease among farmed and ornamental fish can cause significant economic damage to the aquaculture industry (Santos et al., 2017). Previous studies have shown that fish can acquire immunity after exposure to *I. multifiliis.* Immunity of fish against *I. multifiliis* may be related to serum and mucosal-specific immunoglobulins that can detect antigen Ich (Grøntvedt et al., 2003; Sigh et al., 2004; Yu et al., 2018). Fish immunization against white spot disease is one of the safest ways to reduce the risk of suffering fish (Lieke et al., 2020). However, the high cost of providing the vaccine is one of the most critical limitations of controlling white spot disease. Therefore, researchers have been trying to find a new and practical way to produce antigens to increase the acquired immunity in fish in recent years.

A study by Heidarieh et al. (2017) showed that a dose of 170 Gray is an optimum dose of gamma radiation to inactivate trophonts of *I. multifiliis*. They also found that using radiation-inactivated parasites could trigger the fish's immune system to respond (Heidarieh, et al., 2014, 2015, 2021). Therefore, using gamma radiation parasite inactivation to prepare vaccines has become widespread instead of traditional thermal or chemical methods (Myint, 2018). Therefore, this study is designed to increase our knowledge about the use of radiation to inactivate pathogens.

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DOI: https://doi.org/10.22034/ijab.v11i5.1916

Table 1. Chemical composition of the basal diet, commercial pelleted diet (Behparvar, Iran).

Proximate analysis (%)	
Dry matter	89
Crude protein	36-38
Crude lipid	10-12
Ash	10
Fiber	3.5
Phosphorous	0.7

Fish skin shows architectural similarities with that of mammalians, and the fish epidermis is a multilayered tissue that separates from the dermis by a mucus-producer layer. The fish skin shares crucial protective functions, namely against infection using innate antimicrobial defense systems (Henrikson et al., 1967a, b). The digestive tract of fish can be described basically as a muscular tube lined by a mucous membrane of columnar epithelial cells. The GI tract is a major route of infection in fish. Mucosa is critical in digestion, absorption, and metabolic processes, acts as a barrier to pathogenic infections, and prevents both viable and non-viable bacteria (Ringø et al., 2010). Since the skin and intestinal mucosa play an essential role in the innate immunity of fish, the changes in the skin and intestinal epithelium can indicate the fish's immune response to the parasite. Therefore, this study aimed to show whether gamma radiation to *I. multifiliis* trophonts could affect the number of goblet cells and the properties of the skin and gut mucus layers of rainbow trout, *Oncorhynchus mykiss*.

Materials and Methods

Fish: A total of 120 healthy rainbow trout, with an average body weight of 50±15 g, were obtained from a local farm near Karaj, Iran. All fish were examined for *I. multifiliis* infectious. Specimens were randomly placed in 4 polypropylene tanks (300 L, 105×70×77 cm), equipped with aerators containing free-flowing healthy water with the flow rate set at 0.5 l/s, pH 7.8, water temperature 15±1°C and dissolved oxygen 8 ppm. During the adaptation period, fish were fed twice daily at 1.5% body weight/day with a commercial trout pellet diet (Behparvar, Iran) (Table 1).

Parasite trophonts preparation: Maturing live trophonts of *I. multifiliis* were isolated and counted (each vial containing 1000 live trophonts) from an infected fish and used to challenge fish. Also, detached trophonts were assigned to glass aquaria containing aerated tap water at 17-18°C for transforming the trophonts into tomonts. Afterward, the tomonts left overnight for further development into tomocysts and infective theronts (von Gersdorff Jørgensen, 2017). Next, all fish were challenged with the parasite. Exposed and infected fish were clinically examined for general behaviors, changes in color, respiratory manifestation, and any white spots all over the body. The theronts were counted using a calibrated ocular micrometer to prepare vials containing 5000 theronts in each vial (Zhang et al., 2013).

Radiation procedure: Several thousand trophonts of *I. multifiliis* in Phosphate-buffered saline (PBS) were used for irradiation treatment. All irradiations were performed on dry ice with cobalt 60 sources at 170 Gray in gamma cell irradiation instrument Nordian, model 220, with a dose rate of 0.22 Gy/sec and 20469 Ci activities (Heidarieh et al., 2014, 2015).

Formalin inactivation: Live trophonts (each vial containing 1000 live trophonts) were inactivated with 3% formalin and incubated at room temperature for 2 h; treated trophonts were centrifuged at 3000×g for 2 min the supernatant was discarded. The pellets were washed three times with 1 ml of 0.15 M sterile phosphate-buffered saline (PBS, pH 7.4). After the wash, the formalin-treated trophonts were harvested by centrifugation at 3500×g for 3 min (Xu et al., 2009; Heidarieh et al., 2015).

Treatments: Three vials of each of the following parasite trophonts were prepared as follows: (1) Untreated trophonts as the positive control (live theronts), (2) suspended trophonts in 3% formalin (formalin inactive trophonts) and (3) irradiated trophonts with a dose of 170 Gray gamma rays (irradiated trophonts).

A total of 120 parasitic-free rainbow trout were randomly assigned to each treatment, infected, and control group (4 groups, with 10 fish per aquarium, 85×50×40 cm). There were three repetitions per immunized, infected, and control group. Group 1:

Table 2. Means and standard deviations for length (µm), width (µm), and relative numbers of goblet cell density/total enterocytes in the intestine villus tissues of treated, infected, and healthy rainbow trout.

Groups*	First week			Second week		
	Intestine villus length (µm)	Intestine villus width (µm)	Intestine villus goblet cell density/ total enterocytes	Intestine villus length (µm)	Intestine villus width (µm)	Intestine villus goblet cell density/ total enterocytes
1	121.4±15.7	400±65.5	19.43±2.0^{b}	118.5±15.3	382.7±69.4	19.57±5.0^{b}
2	124.3±20.7	417.1±76.5	32.57±6.0^{a}	121.4±19.5	390±83.0	34.71±5.6^{a}
3	121.4±16.7	388.5±55.5	29.14±5.5^{a}	124.3±25.0	405.7±69.0	32.3±10.0^{a}
4	120±22.3	390±75.7	22±9.5.0^{b}	142.9±30.9	394.3±79.1	32.3±6.8^{a}

Data are mean ± SEM. Those within a column superscripted by different letters are significantly different (P<0.05). * 1: Control (Healthy fish/Negative control); 2: Irradiated trophonts with a dose of 170 Gray gamma-ray; 3: Suspended trophonts in 3% formalin; 4: Untreated trophonts as a positive control (infected with live theronts).

Table 3. length (µm), width (µm) and relative numbers of goblet cell density in the pyloric caeca tissues.

Groups *	First week			Second week		
	Pyloric fold length (µm)	Pyloric fold width (µm)	Pyloric fold goblet cell density	Pyloric fold length (µm)	Pyloric fold width (µm)	Pyloric fold goblet cell density
1	108.57±20.3	580±73.7	14.71±5.02^{b}	108.57±19.5	568.5±70.8	15.3±4.3^{c}
2	102.86±11.1	578.57±76.0	28.57±7.9^{a}	112.9±19.8	550.1±108.2	33.43±14.2^{a}
3	105.7±15.1	560±52.1	33.43±3.9^{a}	107.14±22.1	542.9±84.6	36.3±6.3^{a}
4	110±23.8	555.7±93.0	15.43±4.8^{b}	107.14±16.0	568.5±133.4	22.57±8.5^{b}

Data are mean ± SEM. Those within a column superscripted by different letters are significantly different (P<0.05). * 1: Control (Healthy fish/Negative control); 2: Irradiated trophonts with a dose of 170 Gray gamma-ray; 3: Suspended trophonts in 3% formalin; 4: Untreated trophonts as a positive control (infected with live theronts).

uninfected fish, negative control; Group 2: treatment 1 using 100 gamma-irradiated trophonts per 150 g biomass; Group 3: treatment 2 using 100 formalin inactive trophonts per 150 g biomass; Group 4: infected fish (5000 live theronts), positive control. One week after the first immunization, both treatments were repeated. One week after the second treatment, fish were exposed to the 5000 live theronts in the aquaria (except negative and positive control groups). The exposure process was conducted as described by Heidarieh et al. (2015) with slight modifications. Aquaria were equipped with biological filtration; water was monitored daily for quality and temperature (Heidarieh et al., 2015). Diets were fed to fish twice daily at a 1.5% average fish weight per meal.

Sampling: In the first and second weeks after exposure to the live theronts, five fish from each aquarium were gently transferred to a small plastic aquarium and killed quickly with an overdose of MS222 (200 mg/L) (Sigma-Aldrich, Denmark). The skin sample (2.5×4 cm) was removed in the same place from the left side of the mid-body of each fish (below the dorsal fin). After dissection at the midline in the ventral surface, the proximal gut section and pyloric caeca were gently removed. Sterile sharp scalpels were used to cut tissue. Samples were fixed in 10% buffered formalin for 48 h, dehydrated in alcohols and xylene, and then embedded in paraffin. A five-micron subsample was then rehydrated in alcohol and stained with hematoxylin-eosin. The number of goblet cells in the proximal intestine, pyloric caeca, and total goblet density/cm skin epidermis were defined in each group. Also, the length and width of proximal intestinal villi, and pyloric caeca folds and the epidermal thickness of the skin were measured using an ocular lens (Heidarieh et al., 2021).

Statistical Analysis: Analysis of variance was carried out by One-Way ANOVA in SPSS 16.0 software, followed by Duncan's multiple range tests. The differences were considered statistically significant at the P<0.05 level.

Table 4. thickness (µm) and number of goblet cells density/cm in the skin tissues.

Groups*	First week		Second week	
	Skin thickness(µm)	Skin goblet cells density/cm	Skin thickness(µm)	Skin goblet cells density/cm
1	304.3 ±15.11[b]	584.3±98.5[c]	317.1±33.5[c]	557.1±122.8[b]
2	367.7±43.2[a]	1114.3±274.9[a]	370.1±36.5[b]	1092.7±534.1[a]
3	362.7±38.1[a]	938.6±94.94[b]	407.1±75.4[a]	1271.4±303.9[a]
4	355.7±36[a]	1442.9±75.14[a]	374.3±28.7[b]	1428.6±502.3[a]

Data are mean ± SEM. Those within a column superscripted by different letters are significantly different (P<0.05). * 1: Control (Healthy fish/Negative control); 2: Irradiated trophonts with a dose of 170 Gray gamma-ray; 3: Suspended trophonts in 3% formalin; 4: Untreated trophonts as a positive control (infected with live theronts).

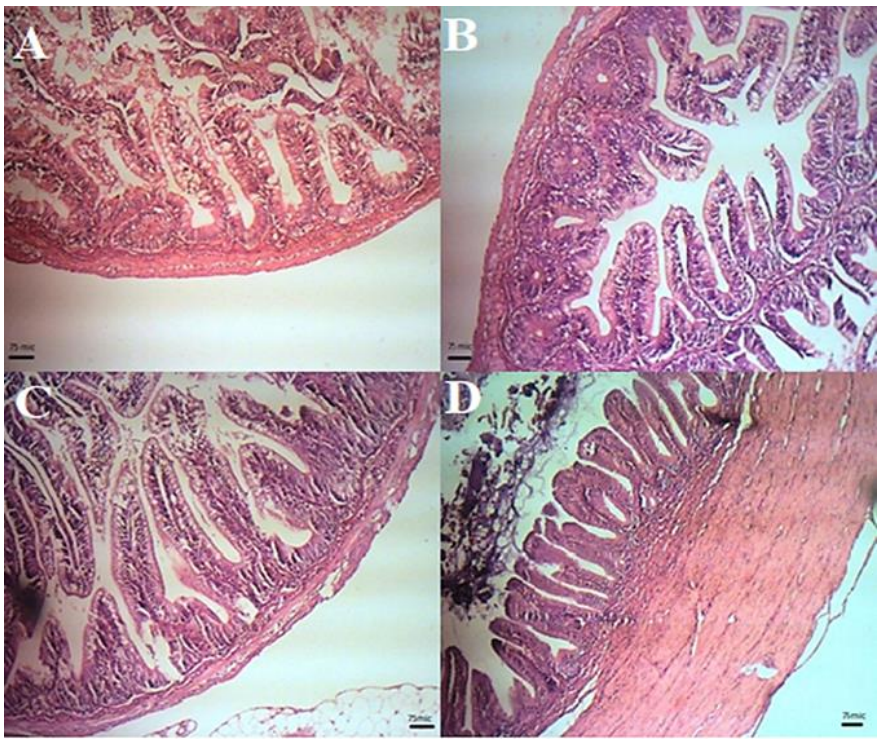

Figure 1. The goblet cell density/total enterocytes of the intestine villus intestine. A: with suspended trophonts in 3% formalin; B: Irradiated trophonts with a dose of 170 Gray-gamma rays; C: Untreated trophonts as positive control (infected with live theronts) D: a normal intestine of rainbow trout (Healthy fish/Negative control) without any treatment (H&E, 200×). The goblet cell/enterocyte hyperplasia of the intestine epithelium was recorded in both of the immunized groups in the first and second week after exposure. In the second week, a higher density of mucous cells/total enterocytes also was seen in infected fish with live trophonts of *Ichthyophthirius multifillis* (positive group).

Results

Alterations in intestine histological parameters were displayed in Table 2, after challenging with *I. multifiliis* at the first and second weeks. No significant differences (P>0.05) were observed between all groups regarding intestine villus length and width. The mucous cells/total enterocytes hyperplasia of the intestinal epithelium was recorded in both treated groups in the first and second week of exposure (P<0.05) (Table 2). During the second week, mucous cells/total enterocyte hyperplasia also was observed in infected fish (positive group) (P<0.05) (Fig. 1).

Microscopic studies showed no significant difference (P>0.05) in fold length and width in pyloric caeca between all treatment groups (Table 3). However, significant increases were found in the mucous cell numbers of the pyloric fold for treated fish by gamma-irradiated and formalin inactive trophonts at the first and second weeks compared to the negative control (P<0.05). The results showed that the pyloric caeca goblet cell numbers significantly increased after two weeks when fish infected fish with live theronts and inactive trophonts (Fig. 2).

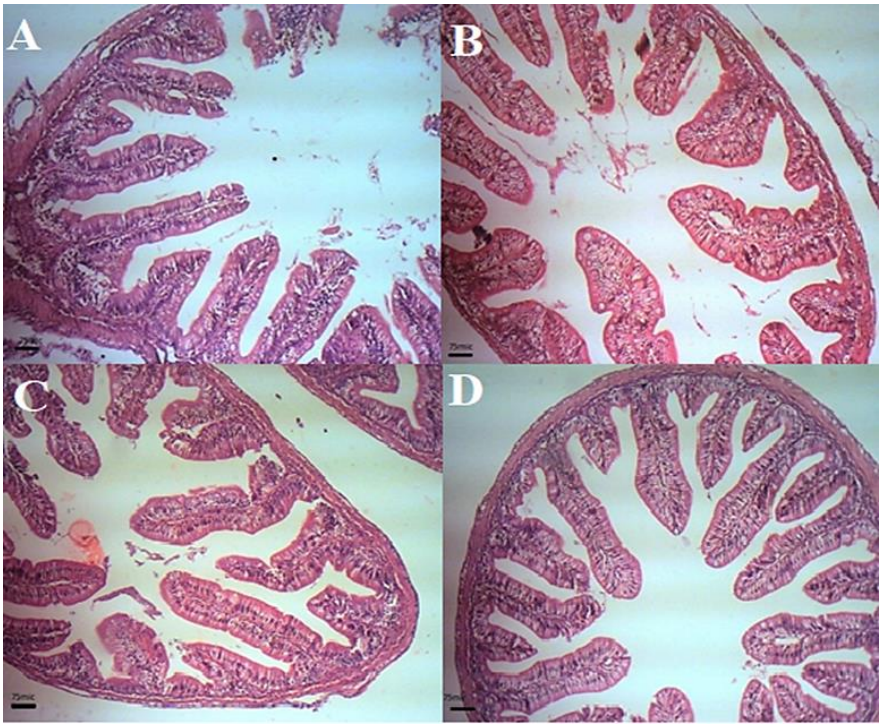

Figure 2. The goblet cell density of the pyloric caeca. A: with suspended trophonts in 3% formalin; B: Irradiated trophonts with a dose of 170 Gray-gamma rays; C: Untreated trophonts as the positive control (infected with live theronts) D: Normal pyloric caeca of rainbow trout (Healthy fish/Negative control) without any treatment (H&E, 200×). The pyloric caeca goblet cell hyperplasia was recorded after two weeks when fish were infected fish with live theronts and inactive trophonts of *Ichthyophthirius multifillis.*

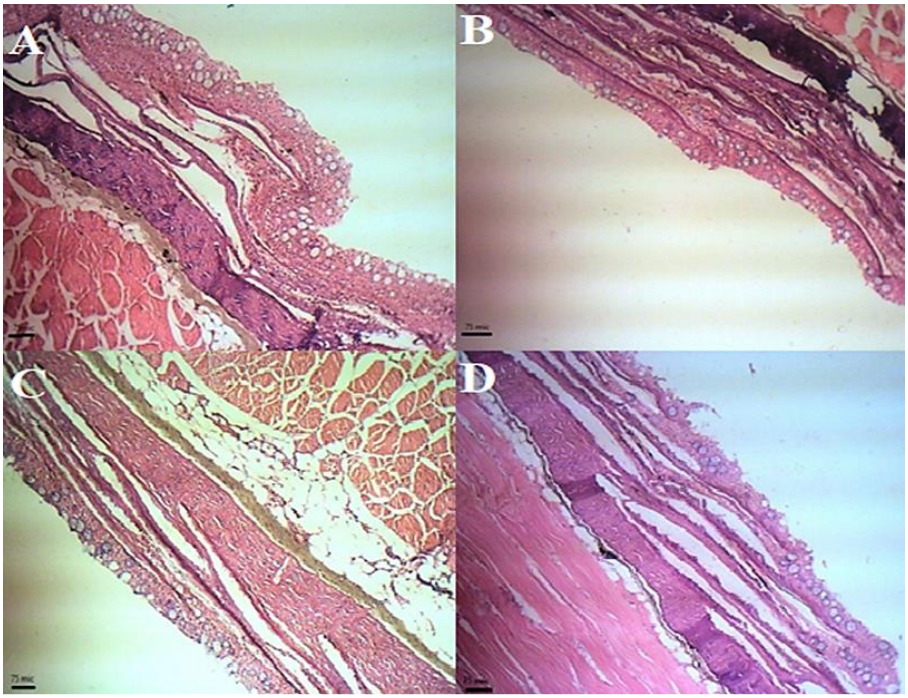

Figure 3. The thickness (µm) and numbers of goblet cells of skin. A: with suspended trophonts in 3% formalin; B: Irradiated trophonts with a dose of 170 Gray-gamma rays; C: Untreated trophonts as positive control (infected with live theronts) D: a normal skin of rainbow trout (Healthy fish/Negative control) without any treatment (H&E, 200×). Significant mucous cell hyperplasia in the skin epidermis was observed after challenging fish with the live and inactive *Ichthyophthirius multifiliis*.

The epidermal thickness of the skin in treated fish with gamma-irradiated and formalin inactive trophonts and infected fish was considerably higher than the negative control group during the first and

second weeks (P<0.05). In addition, there was a marked increase in skin thickness of the treated fish with formalin inactive trophonts among all groups in the second week (P<0.05) (Table 3). Significant changes in the histopathological index, including mucous cell hyperplasia in the skin epidermis, were observed after challenging fish with the live and inactive *I. multifiliis* (P<0.05) (Fig. 3, Table 4).

Discussions

This study investigated how the skin and gut mucus layers respond to theronts of *I. multifiliis* after exposure to either live, gamma-irradiated, or formalin-inactive trophonts in rainbow trout. The findings showed that the number of goblet cells in both treated groups was higher than the negative control fish in the first and second weeks. In contrast, an increase in epidermal thickness was observed in the skin of the treated and infected fish in the first and second weeks. Similarly, a significant increase in skin goblet cells was reported in common carp and catfish exposed to sub-lethal infection of *I. multifiliis* (Cross, 1993). The mucosal cells beneath the epithelial cells of the fish skin are essential for mucosal defenses against ectoparasitic infections (Wells et al., 1990; Zhang et al., 2013). Furthermore, the epidermal thickness shows parasite re-infection and has been proposed to result from a non-specific immune stimulation (Ventura et al., 1985; Holm et al., 2015).

The histological examination showed increases in the mucous cell numbers of the pyloric fold and intestine tissue of immunized and infected fish. The mucous cells contain several anti-parasite compounds, such as lysozymes, lectins, and proteolytic enzymes (Yu et al., 2021). The critical role of mucus is to protect the gut mucus layers against parasites (Schroers et al., 2009). Hansson (2012) reported a significant relationship between mucous cell numbers and various enteric infections caused by parasites.

The current results and supporting previous findings reported by Syahputra et al. (2019) and Heidarieh et al. (2021) indicated that *I. multifiliis* might invade tissues other than the skin and gills of fish (Syahputra et al., 2019; Heidarieh et al., 2021). The main reason is unknown, but it can be attributed to the possible entryways of the parasite into fish peritoneal cavities, including penetration of the pneumatic duct/esophageal wall and migration from the anus into the rectum and through the intestinal wall (Buchmann, 2020). The results of this study are consistent with the study by Buchmann (2020), which showed the detection of trophonts in the abdominal adipose tissue and intestines of infected channel catfish (*Ictalurus punctatus*) exposed to *I. multifiliis.* In another study, *I. multifiliis* could live in the peritoneal cavities of catfish infected with live torphonts (IP) (Dickerson et al., 2014 Buchmann, 2020).

Evaluation of gamma-irradiated and the formalin-inactive trophonts treatments revealed that alteration of skin and gut mucus layer's properties in immunized rainbow trout against *I. multifiliis* of the two treatments was non-significant at the first and second weeks after exposure. Therefore, the present experiment demonstrated the improvement in goblet cell numbers and the properties of the mucus layers in the skin, intestine, and pyloric caeca tissues of the treated fish. Also, we suggest that gamma-irradiated trophonts and formalin inactive trophonts can be safe for use in rainbow trout against *I. multifiliis*.

Acknowledgments

The authors are grateful for the financial support provided by the International Atomic Energy Agency (IAEA), Vienna, Austria, and by the Nuclear Science and Technology Research Institute (NSTRI), Karaj, Iran.

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