JMPHTR 1 (2013): 8-12

Journal of Medicinal Plant and Herbal Therapy Research
www.bluepenjournals.org/jmphtr

The effects of powdered ginger (*Zingiber officinale*) on the haematological and immunological parameters of rainbow trout *Oncorhynchus mykiss*

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Article History
Received 14 January, 2013
Received in revised form 06 February, 2013
Accepted 13 February, 2013

Key words:
Vegetable extracts, Immunology diet, Fish

Full Length Research Paper

ABSTRACT

Immunostimulants are substances which stimulate the specific and/or non-specific defence systems of fish, enhancing resistance to pathogens during stressful periods. This study evaluated the immune-stimulatory effects of dietary powdered ginger rhizome (*Zingiber officinale*), in rainbow trout (*Oncorhyncus mykiss*). The fish were hand-fed with a diet containing 1% powdered ginger rhizome (*Z. officinale*) once a day at 9:00 a.m. for 12 weeks. At the end of the experimental period, hematological parameters including hematocrit (Htc), hemoglobin (Hb), red blood cell (RBC), white blood cell (WBC) and immunological parameters including serum lysozyme activity and respiratory burst activity were determined. The results obtained demonstrate that fishes with the supplement of powdered ginger rhizome showed significant immunostimulatory effect, increase in WBC, Hct, RBC values, respiratory burst activity and lysozyme activity when compared with the control group ($p<0.05$). These results indicate that dietary powdered ginger rhizome stimulates the immune system in the rainbow trout.

INTRODUCTION

Rainbow trout (*Oncorhynchus mykiss*) is the first most commonly cultivated coldwater fish in aquaculture industry of Iran. Due to intensive culture practices for the increased production, disease management continues to pose a serious threat to aquaculture industry. The use of immunostimulants in aqua-feed is considered as a modern and promising alternative to antibiotics and vaccines as a prophylactic measure in intensive aqua-culture. Immunostimulants also have the ability to increase resistance to microbial infections and stressors like handing, transport, grading and poor water quality in cultivated fish (Raa, 2000). The effects of a number of immunostimulants such as levamisole (Siwicki et al., 1990), glucans (Jeney and Anderson, 1993), chitins (Cuesta et al., 2003), vitamin C, lactoferin (Sakai, 1999) as well as various products derived from medicinal plants have been studied concerning their ability to preventing diseases in a variety of fish species. It is well known that these agents facilitate the function of phagocytic cells (Dugenci et al., 2003), increase their bactericidal activities (Dugenci et al., 2003; Secombes and Olivier, 1997), and stimulate the natural killer cells and lysozyme activity (Sakai, 1999; Yin et al., 2006). These agents boost the specific immune response in fish (Christybapita et al., 2007) which confer enhanced protection from infectious diseases. Immunostimulants can also be used as adjuvants, to increase the specific immune response (Secombes and Olivier, 1997; Anderson, 1992). Non-specific immunity plays an especially important role in the defence of fish and is the sole immunological mechanism

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by which invertebrates protect themselves from disease (Secombes and Olivier, 1997). There are many experiments on non-specific immunostimulations of fish that suggest the method has considerable potential for reducing losses in aquaculture, both during larval and on-growing stages. Recently, there is an interest in using medical and aromatic herbs or spices as feed additive in fish diets instead of chemical products, to avoid side effects related to the currently used immunostimulants and the practice in organic aquaculture. Herbal immunostimulants are substances which activate white blood cells (WBC) and may render fishes more resistant to infectious diseases, by the stimulating phagocytic cells as well as complement lysozyme and antibody responses of fish (Secombes and Olivier, 1997). The rhizome of ginger (*Zingiber officinale*) has been reported to possess a broad-spectrum of prophylactic and therapeutic activities (Ernst and Pittler, 2000). Ginger is effective in the control of a range of bacterial, viral, fungal and parasitic diseases (Agrawal et al., 2001; Martins et al., 2001; Endo et al., 1990). In addition, ginger is effective as an immunomodulatory agent in animals and fish and helps to reduce the losses caused by diseases in aquaculture (Nya and Austin, 2009; Ali et al., 2008; Zhou et al., 2006; Tan and Vanitha, 2004).

The aim of the present study was to evaluate the immunostimulant effects of dietary intake of powdered ginger rhizome on immune responses in rainbow trout.

MATERIALS AND METHODS

After two weeks of adaptation of the experimental fishes to a control diet, they were fed with experimental diets containing 1% of powdered ginger rhizome for 12 weeks. At the end of the experimental feeding period, the hematologic and immunologic parameters of the fishes were examined. The present study was performed at the Coldwater Fishes Research Center (CFRS), Tonekabon, Iran.

Preparation of ginger and fish food

Fresh rhizome of ginger (*Zingiber officinale* Rosce) was purchased from a local market in India and authenticated by a botanist from Institute of Medicinal Plants, Jehad-e-Daneshgahi. The plant was dried in the shade. The dried rhizome was crushed into powdered form mechanically and was sieved using a household sifter and then mixed directly with basal fish diet (Biomar) to achieve 0 g (control) and 1 g per 100 g of feed. Afterwards, the feed was steam-pelleted and was cooled until it became dry.

Fish and experimental conditions

For experimental use, 600 rainbow trout of 46±1 g mean initial weight were randomly divided in six 2,000 liter round concrete ponds with a continuous water flow of 5 L per min. Ponds were supplied with well water (temperature: 15±1; O_2: 7.2±0.2 mg L^{-1}; pH=8±0.3). These fishes were randomly distributed in two groups, and three replicates. One group received 1% of powdered ginger rhizome, and the other group of fishes (control) did not receive powdered ginger rhizome with diet. The fishes were hand-fed once a day with diet medicated with 1% powdered rhizome of ginger or placebo at a rate of 2% body weight at 9:00 a.m. for 12 weeks and three times with normal diet.

Bleeding and serum collection

After completion of the feeding trial of 84 days, sampling was carried out for the analysis of blood parameters, respiratory burst activity and serum lysozyme activity. Five fishes from each replicate with a total of 15 fish from each group were rapidly netted and anaesthetized with 50 mg/L of tricaine methanesulfonate (MS_{222}, Sigma Chemical Co. St. Louis, MO, USA). Blood was drawn from the vena caudalis. Half the blood sample was then transferred immediately to sterile penicillin vial containing a pinch of lithium heparin powder, shaken gently and kept at 4°C; and for serum separation, the remaining blood sample was transferred to sterile Eppendorf tubes without anticoagulant and centrifuged at 2000 g for 10 min. The serum was collected with a micropipette and then was stored in sterile Eppendorf tubes at -20°C until used for assay.

Determination of hematological parameters

Blood was analyzed with routine methods adopted in fish hematology (Blaxhall and Daisley, 1973; Ivanova, 1993; Haghighi, 2010). The total red blood cell count (RBC $\times 10^6$/µl) and total leukocyte count (WBC $\times 10^3$/µL) were determined manually using a Neubauer's haemocytometer with Hayem solution as a diluent. Percentage of RBC and WBC were determined by counting 1500 and 200 cells, respectively. The hematocrit percentage was determined in duplicate by using microhaematocrit-heparinized capillary tubes of 75 µL volume and a microhaematocrit centrifuge at 15000 g for 5 min (Goldenfarb et al., 1971). The hemoglobin (g/dL) concentrations were determined by the cyano-methaemoglobin method (Drabkin, 1945) using a hemoglobin reagent set (Ziest Chem Diagnostics). The values of red blood cell indices of mean cell hemoglobin (MCH pg), mean cell hemoglobin concentration (MCHC %), and mean cell volume (MCV fl) were calculated according to Wintrobe (1993). The differential leukocyte count was carried out using blood smears stained with

Wright-Giemsa. The percentage composition of leukocytes was determined based on their identification characters listed by Ivanova (1993).

Determination of immunological parameters

Respiratory burst activity

The nitro blue tetrazolium (NBT) assay was used to determine the respiratory burst activity according to the method of Anderson in 1992. In this assay, the activity of the neutrophils in the NBT test depends on the glass adherence, and the production of oxidative radicals. Briefly, two drops of each blood sample of replicates were dropped immediately on a clean glass coverslip that was placed on a moist paper towel in 60 mm diameter petri dishes and incubated for 30 min. After incubation, neutrophils were adhered to the glass, the excess cells were gently washed with phosphate buffered saline (PBS; pH 6.2) and excess solution was drained off. A drop of 0.2% NBT in PBS solution is placed on top of the remaining (glass-adherent) blood cells and overlaid with a glass microscope slide, incubated for 20 min, and then it was observed microscopically. The cells that took up the blue dye were counted in 3 microscope areas and numbers were compared with untreated control samples.

Serum lysozyme activity assay

In this study, an assay was used based on the lysis of *Micrococcus lysodeikticus* for determining its activity. Serum lysozyme activity was measured according to the method of Ellis (1990). Briefly, 0.03% lyophilized *M. lysodeikticus* in 0.05 mM solution phosphate buffer (pH 6.2) was used as substrate. 10 µL of fish serum was added to 250 µL of bacterial suspension in duplicate wells of a U-bottom microtitre plate and reduction in absorbance at 490 nm was determined after 0.5 and 4.5 min of incubation at 22ºC using a microplate reader. A unit of lysozyme activity was defined as the amount of sample causing a decrease in absorbance of 0.001 per min.

Statistical analysis

Values for each parameter measured were expressed as mean ± standard error of mean. The results were analyzed with one-way analysis of variance (ANOVA). Differences at $p<0.05$ were regarded as statistically significant.

RESULTS

Hematology

The results showed that the groups that received powdered ginger rhizome showed a significant increase in Hct, Hb, RBC, MCHC and a decrease in MCH values ($p<0.05$; Table 1). There were no significant differences between MCV values in the two groups ($p>0.05$; Table 1). Long-term supplementation with 1% powdered ginger rhizome induced an increase in WBC and in neutrophils statistically ($p<0.05$). However, there were no significant differences between lymphocytes and monocytes in the two groups ($p>0.05$).

Immunology

The results indicate that the lysozyme activity and respiratory burst activity were enhanced considerably in the group treated with 1% powdered ginger rhizome in diet compared to the control group. The lysozyme activity was higher in the group fed with 1% powdered ginger rhizome for 12 weeks compared to control group after 0.5 and 4.5 min of incubation ($p=0.032$ and $p=0.038$, respectively; Table 2). The number of NBT- Positive cells increased significantly in the group fed with 1% powdered ginger rhizome compared to control group ($p=0.04$).

DISCUSSION

In aquaculture, the application of dietary medicinal herbs as immunostimulants can elevate the innate defense mechanisms of fish against pathogens during periods of stress, such as, intensive farming practices, grading, sea transfer, vaccination and reproduction. Hematological assays may provide an index of the physiological status of fish. Leucocyte count, erythrocyte count, hematocrit and hemoglobin are particularly recommended as tests that could be performed on a routine basis in fish farms to monitor the health of the stock. The present study indicates that rainbow trout fed powdered ginger rhizome for 12 weeks showed increased haematocrit, haemoglobbin, erythrocyte, MCH, MCHC, WBC values and neutrophils percentage in comparison to the control group ($p<0.05$). De Pedro et al. (2005) indicated that total and differential leukocyte counts are important indices of non-specific defense activities in fish. Also, they are centrally involved in phagocytic and immune responses to bacterial, viral and parasitic challenges (Houston, 1990).

Phagocytosis and the respiratory burst response by phagocytes in blood and tissues present a major antibacterial defense mechanism in fish (Secombes, 1996). Respiratory burst activity measured by NBT is one of the most important bactericidal mechanisms in fish (Secombes and Fletcher, 1992). Staining the neutrophils with the NBT dye helps to confirm their activity. The soluble NBT dye, taken in by pinocytosis into the neutrophils, is reduced to dark blue formazan granules

Table 1. Comparison of erythrocyte and leukocyte profile of rainbow trout fed with 1% ginger of feed.

Indices (Units)	Control	Ginger	Probability
Hematocrit (%)	35±1	49±1	< 0.001
Hemoglobin (g dL^{-1})	9.1±0.4	11.3±0.2	< 0.01
RBC (×10^6 cells mm^{-3})	1.5±0.18	2.3±0.07	< 0.001
MCV (fl)	233.333±11	213.043±9	> 0.05
MCH (pg)	60.66±2	49.13±1	< 0.01
MCHC (%)	16.47±2	23.06±1	< 0.05
Leukocytes (×10^3 cells mm^{-3})	52.3±0.4	56.8±0.5	< 0.001
Lymphocytes (%)	87±3.2	86±5.1	> 0.05
Neutrophils (%)	10±0.3	12±0.1	< 0.01
Monocytes (%)	3±0.2	2±0.8	> 0.05

Table 2. Effect of 1% powdered ginger rhizome in diet on respiratory burst activity (NBT assay) and on lysozyme activity of rainbow trout after 12 weeks feeding trial.

Parameters	Control group	Ginger group	Probability
NBT- Positive cells (in 15 µL of blood)	4.56±0.82	7.39±0.56	0.04
Lysozyme activity after 0.5 min of incubation (IU/ml)	1662±15	1726±13	0.032
Lysozyme activity after 4.5 min of incubation (IU/ml)	1905±23	1984±12	0.038

that are distinctive on microscopic examination. A variety of medicinal herbs are known to stimulate phagocyte cells including ginger, garlic, curcumin and turmeric (*Curcuma longa*), etc, (Dugenci et al., 2003; Nya and Austin, 2009; Nya and Austin, 2011; Behera et al., 2011; Alambra et al., 2012). In the present study, the respiratory burst activity of blood leukocytes was significantly higher than in the control group after 12 weeks ($p<0.05$). This result is consistent with data obtained by Dugenci et al. (2003), who reported that extracellular and intracellular respiratory burst activity and phagocytic activity of leukocytes were enhanced by feeding with aqueous extract of powdered ginger roots in rainbow trout.

Lysozyme activity is an important component in the immune system of fish. Lysozime is an important enzyme in the blood that actively lyses bacterial of cell wall peptidoglycans. It is also known to act as opsonin and activate the complement system and phagocytes (Magnadottir, 2006). In the present study, powdered ginger rhizome incorporated in the diet significantly enhanced the lysozyme activity after 12 weeks. The lysozyme level enhanced with dietary *Cotinus coggyria* (Bilen et al., 2011) and dietary ginger (*Z. officinal*) (Dugenci et al., 2003; Nya and Austin, 2009) in rainbow trout. Therefore, our results are in agreement with the results obtained from mentioned researchers. However, it has been reported elsewhere that no lyzosyme level changes were observed with dietary vitamin C as an immunostimulant in rainbow trout (Verlhac et al., 1995; 1996).

Conclusion

The results of this study indicate that powdered ginger rhizome is able to enhance the non-specific immune response in rainbow trout. However, future studies might look into the dose-response, determination of optimal dose and duration treatment, and its use in large scales in fish farms. In general, this study suggests that ginger can be applied as an alternative diet and a supplement to boost immune system for rainbow trout.

ACKNOWLEDGEMENT

The authors thank Dr. Motalebi and the Management of Iranian Fisheries Research Organization for their financial support and for providing necessary facilities for carrying out this research.

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