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EVALUATION OF IMMUNE RESPONSES IN AN INDIAN CARP, *LABEO ROHITA* (HAMILTON) FED WITH LEVAMISOLE INCORPORATED DIET

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ABSTRACT

Effects of oral administration of levamisole on the selected components of the immune system of an Indian carp, *Labeo rohita*, were evaluated with a view in assessing the potential of using this compound as an immunostimulant in culturing this fish. Sub-adults of *L. rohita* were fed with levamisole incorporated diet (5 mg kg^{-1} body weight), on the first day and every third day for 16 days (6 doses) and effects on haematocrit, total and differential leucocyte counts, phagocytic activity, production of oxygen radicals from phagocytes in the blood, lysozyme activity in the serum, total protein and immunoglobulin levels in the blood plasma were assessed at different time points in comparison to the levels of controls. Results showed that the populations of total leucocytes, neutrophils, monocytes and lymphocytes were elevated significantly on 14 and 21 days post levamisole treatment. In addition, total phagocytic activity, phagocytic index and lysozyme activity of the fish blood were enhanced significantly on 21 days post treatment. Results support the potential use of levamisole as an immunostimulant in culturing this fish.

Keywords: *Labeo rohita*, levamisole, immunostimulant, Indian carp

INTRODUCTION

In fish culture, the use of immunostimulants is opening new opportunities to improve fish health and reduce the losses by diseases and other stressors. Immunostimulatory effects of a number of synthetic chemicals and biological substances for fish have been reported (Anderson, Siwicki & Rumsey, 1995; Sakai 1999; Jeney & Jeney, 2002). Levamisole is an antihelminthic used for the treatment of nematode infections in man and animals (Treves–Brown, 2000). Levamisole has been reported to be effective immunostimulant for common carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*), gilthead seabream (*Sparus aurata*) and Atlantic salmon (*Salmo salar*) (Siwicki, 1987; Mulero, Esteban, Munoz & Meseguer, 1998; Sakai, 1999; Findlay & Munday, 2000).

Labeo rohita (rohu), an Indian carp is a highly commercial edible fish species cultured in the Asian region. It has been used for stocking reservoirs and in polyculture systems (Pillay, 1990). *L. rohita* is one of the exotic fish species currently used in culture based fishery in inland reservoirs of Sri Lanka. *L. rohita* stocks are maintained in freshwater fish breeding stations of the National Aquaculture Development Authority (NAQDA) of Sri Lanka for continuous seed production for various culture systems. However these fish maintained in the breeding stations are highly susceptible to parasitic and bacterial infections. In the present study, effect of administration of levamisole through feed on selected components of the immune system of *L. rohita* were determined with a view in evaluating the potential of using this compound as an immunostimulant in *L. rohita* culture.

MATERIALS AND METHODS

Fish

Sub-adults of *L. rohita* weighing 200 - 300 g body weight (BW) were obtained from the Udawalawa Fish Breeding Station, NAQDA, Sri Lanka and transported to University of Kelaniya. Fish were maintained in outdoor cement tanks filled with aged tap water with continuous aeration for 4 weeks. During the acclimation period, fish were fed daily with commercial fish food pellets (Prima,

Colombo, Sri Lanka) at 1% of the BW. Water temperature during the acclimation period ranged from 27 to 28ºC.

Fish feed

A fish feed was prepared by first mixing dry ingredients, rice bran (4%), corn flour (2%), soybean powder (53.33%) and fish meal powder (26.6%) together for 15 min to ensure homogeneity and then adding a pulp of wheat flour (13%) made in hot water. This mixture was steamed for 15 min, allowed to cool, the vitamin and mineral premix (1%) and soybean oil (40 ml kg^{-1}) were added, mixed well and palletized. Control diet was prepared using the above procedure without adding levamisole. For levamisole incorporated diet, the feed was prepared using above ingredients following the same procedure and levamisole (Sigma, St Louis, MO, USA) at 0.05%, dry weight basis was added to steamed and cooled feed mixture and then palletized. The control pellets and levamisole incorporated pellets were separately dried in the oven at 40°C and stored in tightly sealed plastic bags at 8-10°C until they were used in the feeding experiments.

Levamisole treatment

Sub-adults of *L. rohita* (278 ± 12 g) were introduced to two sets of cement tanks (n=8 per tank) each filled with 1875 L aged tap water with continuous aeration. Fish in two tanks were fed with levamisole incorporated diet, on the first day and every 3rd day for 16 days (6 doses, two divided doses per day) at 5 mg levamisole kg^{-1} body weight and fed with the control diet for the remaining days. Fish in the other two tanks (control fish) was fed daily two times with comparable rations of the control formulated diet. Half of the water in each of the tanks was exchanged with fresh aged tap water every four days.

Blood sampling

Blood samples were collected from four fish from each tank at 14 and 21 days after the last application of levamisole for immunological assays. Blood samples were taken from the fish by bleeding from caudal vein. Fish were anaesthetized in neutralized benzocaine (200 mg L^{-1}) prior to blood sampling (Treves-Brown, 2000).

Haematocrit and leucocyte counts

For determination of the haematocrit levels, blood samples were taken into heparinized capillary tubes and centrifuged in the haematocrit centrifuge. Percentage of erythrocytes was measured using the haematocrit gauge. Total leucocyte count was determined using Shaw's solutions as dilution fluids following the method of Hesser (1960). Blood smears of the fish were prepared, fixed in 100% methanol and were stained with Wright - Giemsa stain. Different types of leucocytes were identified as described by Hibiya (1982).

Phagocytosis assays

Phagocytic cells were detected using *Staphylococcus aureus* (Sigma, St Louis, MO, USA) as described by Anderson & Siwicki (1995). A sample (0.1 mL) of blood was placed in a microtiter plate well, 0.1 mL of *Staphylococcus aureus* 1×10^7 cells suspended in phosphate buffered saline pH 7.2, was added and then mixed well. The bacteria–blood solution was incubated for 20 min at room temperature. Five µL of this solution was taken on to a clean glass slide and a smear was prepared. The smear was air dried, then fixed with ethanol (95%) for 5 min and air dried. Then the smear was stained with Giemsa stain for 10 min. The two smears were made from each fish. The total of 100 neutrophils and monocytes from each smear were observed under the light microscope and the number of phagocytizing cells and the number of bacteria engulfed by the phagocyte were counted. Phagocytic activity and phagocytic index were calculated as follows: Phagocytic activity equals the number of phagocytizing cells divided by the total number of phagocytes counted. Phagocytic index is expressed as the total number of bacteria engulfed by the phagocytes, divided by the total number of phagocytes containing engulfed bacteria.

Nitroblue tetrazolium assay

Production of oxygen radicals from phagocytes in the blood was measured using nitroblue tetrazolium (NBT) dye as described by Anderson & Siwicki (1995). A sample (0.1 mL) of heparinized blood was placed in to a microtiter plate well and equal amount of 0.2% NBT (Sigma, St Louis, MO, USA) was added, the NBT-blood cell suspension was incubated for 30 min at room

temperature. A sample (0.05 mL) of the NBT-blood cell suspension was taken out and added to a glass tube containing 1.0 mL of N,N-dimethylformamide solution. Then the mixture was centrifuged for 5 min at 3000g. The supernatant was taken into a glass cuvette and absorbance was read at 540 nm using a spectrophotometer.

Lysozyme assay

Lysozyme activity of blood serum was determined as described by Anderson & Siwicki (1995) with some modifications. Blood serum was prepared by centrifuging the blood at 3000 g for 5 min. Serum (0.1 mL) was placed in test tubes and 0.9 mL of a 0.75 mg mL^{-1} *Micrococcus lysodeikticus* (Sigma, St Louis, MO, USA) suspension in phosphate buffered saline, pH 6.2 was added and mixed well. The absorbance was measured at 450 nm by a spectrophotometer at 1 min intervals for 10 min after mixing with bacteria and rate of change of absorbance calculated. Lysozyme activities were calculated using hen egg white lysozyme (Sigma-Aldrich) as a standard.

Total protein and total immunoglobulin in plasma

Total protein content in blood plasma was determined using Peterson's modifications of the micro-Lowry method using a protein assay kit (Sigma Diagnostics P 5656, Sigma, St Louis, MO, USA). The protein concentrations were determined using a calibration curve prepared using bovine serum albumin as the standard (50 – 400 µg mL^{-1}). For the determination of the immunoglobulin in the plasma, immunoglobulins were separated from the plasma by precipitation with polyethylene glycol as described by Anderson & Siwicki (1995). Plasma (0.1 mL) was placed in plastic serum vial and 0.1 mL of 12% polyethylene glycol was added and incubated at room temperature for 2 h under constant mixing. After incubation, the solutions were centrifuged at 7000 g for 10 min. The protein content in the supernatant was determined using protein assay kit. The total immunoglobulin content was determined by subtracting the protein content in the supernatant from the total protein content in the plasma.

Statistical analysis

Data are presented as mean ± standard error of the mean for 6 - 8 fish per group. For each parameter, the data obtained for levamisole treated fish were compared with that for the respective control fish using Student's t-test at the significance levels of $P < 0.05$ (Zar, 1999).

RESULTS

All control and levamisole treated fish survived during the experimental period. The haematocrit levels and abundance of populations of total leucocytes and different types of leucocytes in the blood of control fish and levamisole treated fish are presented in Table 1. No significant difference in the haematocrit levels in the blood was found between levamisole treated fish and respective control fish. Total leucocyte counts of levamisole treated fish were significantly higher than those of the respective control fish. The leucocyte count increased by two folds compared to the respective controls.

In the blood smears of *L. rohita*, neutrophils, monocytes, lymphocytes and thrombocytes were recognized in addition to erythrocytes. Thrombocytes were excluded when estimating the leucocyte counts. Abundance of populations of neutrophils, monocytes and lymphocytes in the blood of fish increased significantly after levamisole treatment (Table 1). On the 14^{th} day of the post treatment, abundance of neutrophils and lymphocytes increased nearly by two folds whereas populations of monocytes in the blood of levamisole treated fish increased by 13-fold compared to the respective controls. Populations of neutrophils and lymphocytes in the blood of levamisole treated fish remained increased by nearly two folds even on the 21^{st} day of post levamisole administration. However abundance of monocytes in the blood of levamisole treated fish increased only by about 11-fold compared to the respective controls at 21 days after levamisole treatment.

Total phagocytic activity, phagocytic index, NBT assay, lysozyme, total protein and immunoglobulin levels in the blood of *L. rohita* are presented in Table 2. Phagocytic activity and phagocytic index did not differ significantly

between levamisole treated and respective control fish 14 days after the treatment. However the levels were elevated significantly in levamisole treated fish compared to that of the respective control fish 21 days after the treatment. No significant differences were found between NBT activities in the blood of the levamisole treated fish and respective control fish after the treatment. The activity of lysozyme in the serum was significantly higher in levamisole treated fish compared to the respective controls at 21 days after the levamisole treatments. No significant differences in total protein levels and total immunoglobulin levels in the plasma were found between levamisole treated fish and respective controls in the present study even though the immunoglobulin levels were elevated in levamisole fed fish.

DISCUSSION

In the present study, no significant difference was found between levamisole treated *L. rohita* and respective control fish for haematocrit values, corroborating the findings of Anderson *et al.* (1995) and Findlay & Munday (2000) for *Oncorhynchus mykiss* for *Salmo salar* respectively. However transiently lower haematocrit values have been shown in gilthead seabream (*Sparus aurata*) fed diets containing higher levels (125-500 mg kg^{-1} BW) of levamisole (Mulero *et al.*, 1998).

In this study, oral administration of levamisole through feed had increased total leucocytes, neutrophils and lymphocytes by two fold in *L. rohita* whereas monocyte populations elevated by 11-13 fold. Monocytes and neutrophils which are the main cells of the non-specific defence system are phagocytic and capable of killing a variety of pathogens including bacteria.

Table 1. Effect of oral administration of levamisole* through feed on haematocrit and total and different Leucocyte populations in the blood of sub-adults of *Labeo rohita*

Parameter	14 days after the treatment		21 days after the treatment	
	control	levamisole treated	control	levamisole treated
Haematocrit (%)	33. 7 ± 1.0	34.6 ± 1.3	30.3 ± 1.1	32.1 ± 1.4
Leucocytes (cells mm^{-3})	1758 ± 310	3801 ± 256** (216%)	3034 ± 471	6406 ± 744** (211%)
Neutrophils (cells mm^{-3})	651 ± 53	1654 ± 106** (254%)	1233 ± 234	2675 ± 450** (217%)
Monocytes (cells mm^{-3})	9.5 ± 6.4	125 ± 48** (1316%)	4.6 ± 2.9	51.8 ± 10** (1126%)
Lymphocytes (cells mm^{-3})	1100 ± 267	2023 ± 192** (184%)	1795 ± 314	3686 ± 634**(205%)

* Six doses of levamisole at 5mg kg^{-1} body weight on the first day and every 3rd day for 16 days. Blood samples were taken on 14 and 21 days after the last dose. Data are presented as mean ± SEM of 6-8 fish per group.

** Significantly different from respective control value (Students't test, $P < 0.05$). Percentage values in parentheses indicate the % of the control value.

Table 2. Effect of oral administration of levamisole* through feed on phagocytosis, lysozyme activity, protein and immunoglobulin levels in the blood of sub-adults of *Labeo rohita*

Parameter	14 days after the treatment		21 days after the treatment	
	control	levamisole treated	control	levamisole treated
Phagocytic activity (%)	54.8± 7.7	66.9 ± 4.2	59.5 ± 4.4	73.1 ± 2.5** (123%)
Phagocytic index	1.34 ± 0.07	1.53 ± 0.06	1.43 ± 0.03	1.78 ± 0.07** (124%)
NBT activity(OD at 540 nm)	0.39 ± 0.06	0.51 ± 0.09	0.26 ± 0.09	0.31± 0.07
Lysozyme activity (µg ml^{-1})	5.8 ± 3.8	6.4 ± 2.9	5.8 ± 3.2	29.5 ± 4.2** (508%)
Total protein (mg ml^{-1})	21.8 ± 3.7	29.4 ± 3.8	21.9 ± 4.1	25.4 ± 3.8
Total immunoglobulin (mg ml^{-1})	8.1 ± 3.2	13.9 ± 1.8	7.4 ± 2.4	14.9 ± 2.9

* Six doses of levamisole at 5mg kg^{-1} body weight on the first day and every 3rd day for 16 days. Blood samples were taken on 14 and 21 days after the last dose. Data are presented as mean ± SEM of 6-8 fish per group.

** Significantly different from respective control value (Students' t test, $P < 0.05$). Percentage values in parentheses indicate the % of the control value.

The primary function of fish lymphocytes seems to be to act as the cells of specific immune system via antibody production (Evelyn, 2002). Hence enhancement of immune responses could be expected in *L. rohita* fed with levamisole through its stimulatory effects on different leucocyte populations. Siwicki (1987) reported that *Cyprinus carpio* injected with levamisole showed increased leucocyte numbers, enhanced phagocytic acitivity, and myeloperoxidase activity in neutrophils and elevated lysozyme levels. Oral administration of levamisole has also increased the number of leucocytes, lysozyme levels in the serum and the phagocytic index and NBT reduction by phagocytes in carp (Siwicki, 1987).

In the present study, phagocytic activity and phagocytic index were significantly elevated in *L. rohita* fed with levamisole than the respective control fish on 21 days post-levamisole treatment. In seabream, Sparus *aurata* leucocyte functions including phagocytosis have also been enhanced by dietary intake of high levels of levamisole (Mulero *et al.* 1998). It is known that oxygen free radicals are produced by fish phagocytes during the respiratory burst (Evelyn 2002). The production of oxidative radicals (detected by NBT) was enhanced in *Cyprinus carpio* fed with levamisole (Siwicki, 1989). In the present study, NBT activity in the blood of *L. rohita* rose after the oral treatment of levamisole but there was no significant increase when compared to the controls probably due to high variation. Sahoo, Kumari & Mishra (2005) recorded physiological normal range of some non-specific immune responses in juveniles of Indian major carps and found a wide variation among the individuals within *L. rohita* in the range of immune parameters studied including NBT activity in the blood.

Lysozyme is found in a wide range of vertebrates including fish and is one of the defensive factors against invasion by microorganisms (Evelyn, 2002). In the present study, serum lysozyme levels of *L. rohita* fed with levamisole incorporated diet were significantly elevated compared to the controls on the 21st day after the administration of the last dose. Findlay & Munday (2000) found that bath treatment of fish with levamisole can induce increased activities of both mucus and serum lysozyme of Atlantic salmon, *Salmo salar*. Immunoglobulins

are a major humoral component of the specific immune system. The total immunoglobulin level in the plasma of *L. rohita* fed with levamisole was increased with respect to control fish but the differences were not significant. Total protein in the plasma was not affected by the levamisole treatment.

Challenge experiments have shown that levamisole increased protection against pathogenic bacteria in carp and trout especially through activation of the non-specific defense mechanisms (Anderson *et al.* 1995; Sakai, 1999). This study provides evidence that some components of the immune system in *L. rohita viz.* leucocyte counts, phagocytic activity and phagocytic index in the blood and lysozyme activity in the serum could be enhanced by feeding the fish with levamisole incorporated diet. Incorporation of levamisole into feed holds a potential for immune enhancement in *L. rohita* thereby increasing the resistance of the fish to diseases and stress which may reduce fish mortality rates and offer economic benefits.

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