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A Study on the Effects of Levamisole on the Immune System of Rainbow Trout (*Oncorhynchus mykiss*, Walbaum)

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Abstract: The effects of levamisole on the specific and non-specific immune mechanisms of rainbow trout (*Oncorhynchus mykiss*, Walbaum) were examined. The weight of the 60 fish used (30 in the control group and 30 in the experimental group) in this study was 99.4-216.0 g. After injecting 5 mg kg⁻¹ of levamisole intraperitoneally, blood was taken from the heart of 30 anesthetized fish and hematocrit, leucocrit, leucocyte level, glass-adherent Nitroblue tetrazolium (NBT) positive cell activation, NBT activities, phagocytic activity, potential killing activities of neutrophil and monocytes, myeloperoxidase production (MPO), immunoglobulin, lysozyme and ceruloplasmin (Cp) activity were determined on days 3, 7, 10 and 14. The same procedure was conducted on a control group. No differences were found in the levels of hematocrit, leucocrit or immunoglobulin between the control and experimental groups ($P > 0.05$). However, there were considerable increases in the other immune parameters and significant differences were detected between the control and experimental groups ($P < 0.05$, $P < 0.01$, $P < 0.001$).

Key Words: Immunomodulation, immunity, levamisole, rainbow trout

Levamisol'ün Gökkuşığı Alabalığının (*Oncorhynchus mykiss*, Walbaum) İmmun Sistemine Etkisinin Araştırılması

Özet: Bu çalışmada; levamisolün gökkuşığı alabalığının (*Oncorhynchus mykiss*, Walbaum) spesifik ve non spesifik savunma mekanizmalarına olan etkileri incelendi. Araştırmada; ağırlıkları 99,4-216,0 g olan 60 adet gökkuşığı alabalığı kullanıldı. Balıklara 5 mg kg⁻¹ levamisolün intraperitoneal uygulamasından 3, 7, 10 ve 14. gün sonra kalpten alınan kanda hematokrit, lökokrit, lamele fikse olmuş pozitif hücre aktivasyonu, lökosit düzeyi, NBT aktivitesi, nötrofil ve monositlerin potansiyel öldürme aktiviteleri, fagositik aktivite, myeloperoksidaz üretimi (MPO), immunoglobulin, lizozim ve seroluplazmin aktiviteleri tespit edildi. Aynı işlem kontrol grubuna uygulandı. Çalışmada; hematokrit, lökokrit ve immunoglobulin düzeylerinde, kontrol ve deneme grubu arasında farklılığın olmadığı ($P > 0,05$), ancak diğer immun parametrelerde artışın yüksek olduğu ve kontrol grubu ile deney grubu arasında anlamlı farklılık olduğu görüldü ($P < 0,05$, $P < 0,01$, $P < 0,001$).

Anahtar Sözcükler: İmmunomodulasyon, bağıışıklık, levamisol, Gökkuşığı alabalığı

Introduction

Fish culture is an important and developing industry worldwide. However, intensive fish stocking in ponds affects the health of fish. Consequently, the physiological condition of cultured fish will be affected by environmental conditions. Thus, fish farmers have to practice careful husbandry techniques (1).

For all vertebrates, non-specific defense mechanisms are very important. Since many fish have short lifespans and live in cool water environments, which slow the development of the specific immune response, the non-specific defense mechanisms are more important than the

potentially specific immune response when a fish initially encounters a pathogenic microorganism (2).

Antibiotics, drugs and chemicals have been used for treating fish disease caused by environmental stress and other factors for years (3). However, these are often effective for only a short time and may accumulate in the environment. In the past, the immunological approach to preventing fish disease has been by vaccination against specific pathogens, where vaccines were used for treating a particular disease (2).

The non-specific defense mechanisms are quickly activated by the immunostimulants and are rapidly

prepared to protect the fish against pathogens (2). Recently, there has been increasing interest in the modulation of the non-specific immune system of fish as both a treatment and prophylactic measure against disease (4). In order to determine the positive effects of immunostimulants on specific and non-specific immune systems of fish during treatment period, studies on glucan (5), FK-565 (6), lactoferrin (7), levamisole (8,9) and MDP (10) have been carried out.

Muramyl dipeptide, Freud's complete and incomplete adjuvants, FK-595 and newer immunostimulants such as levamisole have been added to vaccines with promising results (2,8,11-13).

In general, immunostimulants comprise a group of biological and synthetic compounds that enhance the non-specific defense mechanisms of animals (2). Levamisole is a levo-isomer of tetramisole (4). Previous studies have suggested that levamisole treatment leads to an enhanced state of resistance to various kinds of infections (4,14-16).

The present study was conducted to determine the effects of levamisole (first introduced (17) as a broad spectrum anthelmintic and since used extensively and safely in veterinary and human medicine), on the non-specific defense system of rainbow trout. By using this kind of substrate, loss of valuable fish species caused by pathogens in fish culture may be prevented and there may also be an economic benefit for fish farming.

Materials and Methods

Healthy *Oncorhynchus mykiss* (n = 60), weighing between 99.4 and 216.0 g, were obtained from the Fisheries Research Unit of Firat University, Elazığ, Turkey, which was where the experiments were performed. The fish were kept in tanks 1.90 m in length, 0.50 m in width and 0.50 m in depth, with water recirculation. The water was maintained at 16 ± 1 °C and pH 7.8 ± 0.1 and dissolved O₂ 9.3 ± 0.5 ppm. The fish were fed twice daily, 7 days a week. The fish were divided into 2 groups, with 30 in each group. After a 1 week acclimation period, the fish were anesthetized in 50 ppm benzocaine solution. The experimental group was injected intraperitoneally with 0.2 ml PBS + 5 mg kg⁻¹ levamisole and the control group with 0.2 ml phosphate buffer saline (PBS, pH 7.2) (18). Blood was taken from the heart of anesthetized fish to determine levels of

hematocrit, leucocrit and leucocyte, glass-adherent NBT positive cell activation, NBT activities, phagocytic activity, potential killing activities of neutrophil and monocytes, myeloperoxidase (MPO) production, immunoglobulin, lysozyme and ceruloplasmin activity on days 3, 7, 10 and 14 post-treatment by using the methods in Siwicki and Anderson (19), Siwicki et al. (2) and Findlay and Munday (4).

Heparinized hematocrit capillary tubes were filled to the red line. The tubes were then centrifuged for 1 min on a hematocrit centrifuge. The percentages of erythrocyte (hematocrit) and leucocyte (leucocrit) volume were calculated by overlaying the tubes on a sliding scale hematocrit reader.

Total leucocyte numbers were calculated from hemocytometer counts. Leucocytes were fixed and stained using Natt-Herrick solution.

NBT-glass adherent assays for determining neutrophil activity were performed by placing single drops of blood (0.1 ml) on 2 glass coverslips. The coverslips were incubated for 30 min at room temperature. The coverslips were then gently washed with PBS. Drops (0.1 ml) of 0.2% NBT were placed on a microscope slide and the coverslips placed on top so the adherent cells could be incubated for another 30 min with the NBT solution. The activated neutrophils were counted under a microscope at x400.

For the detection of NBT activity by spectrophotometric assay, 0.1 ml of blood was put into a microtiter plate well, and then an equal amount of 0.2% NBT solution was added. After incubation at room temperature for 30 min, 0.05 ml of the NBT-blood cell suspension was removed and added to a glass tube containing 1.0 ml of N, N dimethyl formamide. After centrifugation, reading in a spectrophotometer at 620 nm in a 1.0 ml cuvette was performed.

The heparinized blood was immediately used for the phagocytic assay. Briefly, 1×10^7 cells of *Staphylococcus* sp. in 0.1 ml of PBS were added to 0.1 ml of blood samples in a microplate and incubated for 30 min after thorough mixing in the well. After incubation, the plate was mixed gently and 0.05 ml of this suspension was smeared on the glass slide. After air-drying, the smears were fixed in ethanol, and cells and phagocytosed bacteria were counted.

For the detection of intracellular superoxide radical production, 0.1 ml of blood leucocyte solution was mixed

with an equal volume of 0.2% NBT solutions in PBS containing 0.1 ml of EMEM – 0.1 (Eagles Minimal Essential Media with 0.1% fetal calf sera) in microtiter plates in duplicate. After incubation at room temperature for 60 min, the mixture was mixed with NBT solution containing 1×10^8 *Yersinia ruckeri* cells. The plates were centrifuged at 150 g for 5 min and supernatants discharged. Cells were then washed twice in 70% methanol. After the final washing, the methanol was removed. The optical density (OD) of the samples was read at 620 nm against a KOH/DMSO blank in a spectrophotometer.

Total myeloperoxidase production of leucocytes was detected using the Sigma Kit 390-A.

Total circulating antibody titers were determined by the Biuret method, which detects the amount of nitrogen in amino acids. Plasma (0.1 ml) was mixed with an equal volume of polyethylene glycol in microtiter plates. After incubation at room temperature for 120 min with regular mixing, the plates were centrifuged at 5000 g for 5 min. The OD of the samples was read at 650 nm in a spectrophotometer.

Then 0.5 ml of suspension of *Micrococcus lysodecticus* was added to 0.1 ml of plasma and the concentration of lysozyme activity was determined by spectrophotometric methods at 450 nm.

In the analysis of Cp activity, to freshly prepared serum was added 0.1% substrate in acetate buffer at pH 5.2, containing 0.02% sodium azide. After 15 min incubation, the Cp activity was measured colorimetrically using a spectrophotometer.

Differences in immunological parameters between experimental and control fish were analyzed by Student's t-test using Minitab Statistical Software Release 10.

Results

Levels of parameters of specific and non-specific defense system in the control group and levamisole-injected fish group are given in Tables 1 and 2 and Figures 1-7. All values of the parameters examined increased on the third day post-injection and continued until the end of the experiment. The hematocrit and leucocrit showed no statistically significant differences with levamisole injection ($P > 0.05$) (Table 1, Figures 1, 2). However, the leucocyte numbers were increased significantly ($P < 0.05$, $P < 0.001$) in the levamisole-injected fish (Table 1, Figure 3). The number of cells in adherent NBT positive cell activation was 177 for the levamisole-injected fish group, but it was only 39 for the control group. NBT activity in the levamisole-injected group was higher ($P < 0.001$) than that in the control group (Table 2, Figures 4, 5). In the blood leucocytes isolated from the levamisole treatment group there was an increase in phagocytic activity. Although there were only small differences, the experimental group showed a statistically significant increase in phagocytic ability (Table 2, Figure 4). In phagocytic activity, the maximum numbers of observed cells were 83 and 39 for the experimental and control groups, respectively. The blood leucocytes from fish injected with levamisole showed respiratory burst activity for all the assayed times although the increase ($P < 0.05$) was only statistically

Table 1. Effects of levamisole on hematocrit, leucocrit, leucocyte levels and glass- 2 adherent activities of rainbow trout. Data were analyzed by Student's t – test. Results are expressed as mean \pm standard deviation (S.D.).

Days after injection	Fish groups	Hematocrit (%)	Leucocrit (%)	Leucocyte (x 1000)	Glass-adherent (number)
3	Control	32.22 \pm 1.30	2.30 \pm 1.33	37.63 \pm 3.25	29.20 \pm 2.68
	Experimental	32.80 \pm 1.83	2.50 \pm 1.78	40.80 \pm 4.69	82.60 \pm 10.89***
7	Control	30.33 \pm 1.03	2.00 \pm 0.89	37.30 \pm 1.28	27.80 \pm 3.49
	Experimental	30.83 \pm 3.86	2.33 \pm 1.21	46.35 \pm 8.31	121.0 \pm 4.76***
10	Control	31.00 \pm 1.24	1.90 \pm 0.73	37.77 \pm 1.17	28.00 \pm 8.42
	Experimental	32.10 \pm 2.02	1.90 \pm 0.73	49.90 \pm 5.34*	154.8 \pm 6.09***
14	Control	30.50 \pm 1.50	2.00 \pm 0.81	37.86 \pm 1.09	29.4 \pm 6.69
	Experimental	33.10 \pm 1.37	2.10 \pm 0.87	85.96 \pm 5.07***	153.2 \pm 29.65***

** P < 0.01 *** P < 0.001

Table 2. Effects of levamisole on NBT, phagocytic activity, neutrophil and macrophage potential killing activity, MPO, Total Ig2 Lysozyme and ceruloplasmin activity. Data were analyzed by Student's t – test. Results are expressed as mean ± standard deviation (S.D.).

Days after injection	Fish groups	NBT (ml mg ⁻¹)	Phagocytic activity (number)	Potential killing activity	MPO (number)	Ig (ml mg ⁻¹)	Lysozyme (ml mg ⁻¹)	Ceruloplasmin (% mg)
3	Control	1.30 ± 0.08	34.44 ± 3.20	0.40 ± 0.03	89.80 ± 3.03	20.52 ± 1.47	0.90 ± 0.05	15.05 ± 0.80
	Experimental	1.65 ± 0.10***	59.20 ± 4.20***	0.59 ± 0.08*	102.0 ± 5.78**	21.10 ± 2.18	1.37 ± 0.03***	19.80 ± 1.75**
7	Control	1.30 ± 0.12	35.00 ± 3.00	0.42 ± 0.05	90.20 ± 5.06	21.80 ± 1.38	0.92 ± 0.03	15.16 ± 1.03
	Experimental	1.87 ± 0.10***	69.20 ± 4.49***	0.61 ± 0.07*	120.6 ± 3.90**	21.22 ± 2.54	1.50 ± 0.12***	21.05 ± 0.89***
10	Control	1.30 ± 0.15	33.60 ± 4.27	0.42 ± 0.06	89.60 ± 3.43	21.06 ± 2.19	0.92 ± 0.04	15.48 ± 1.14
	Experimental	2.18 ± 0.77***	78.8 ± 3.49***	0.60 ± 0.07*	122.2 ± 5.61**	21.10 ± 2.77	1.12 ± 0.06***	20.39 ± 1.34***
14	Control	1.31 ± 0.10	35.20 ± 2.58	0.41 ± 0.03	88.20 ± 3.96	21.14 ± 1.45	0.93 ± 0.03	15.16 ± 1.52
	Experimental	2.24 ± 0.73***	71.20 ± 3.36***	0.63 ± 0.04***	132.4 ± 2.44**	22.04 ± 1.78	1.03 ± 0.04**	20.38 ± 1.46***

*P < 0.05 ** P < 0.01 *** P < 0.001

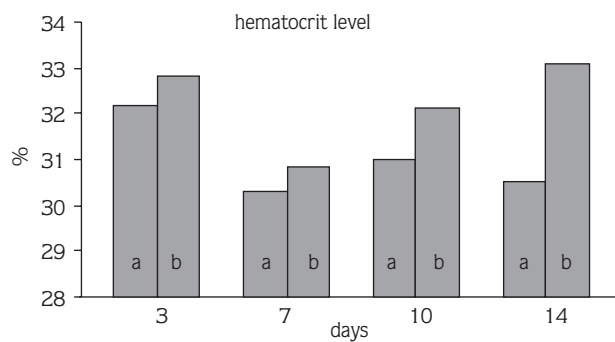


Figure 1. Hematocrit value of rainbow trout 3, 7, 10 and 14 days after the last administration of levamisole. a: Control group, b: Experimental group.

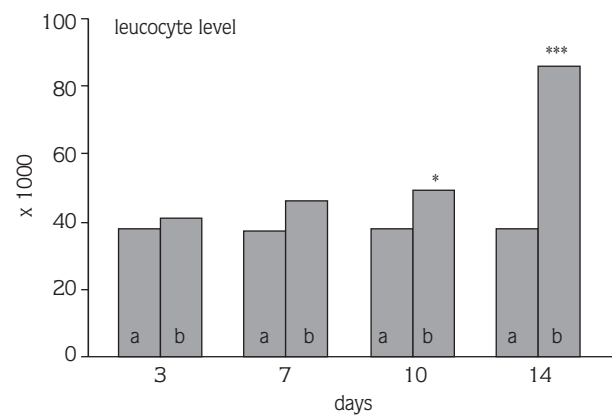


Figure 3. Changes in leucocyte numbers of rainbow trout injected with levamisole a: Control group, b: Experimental group. * P < 0.05 *** P < 0.001.

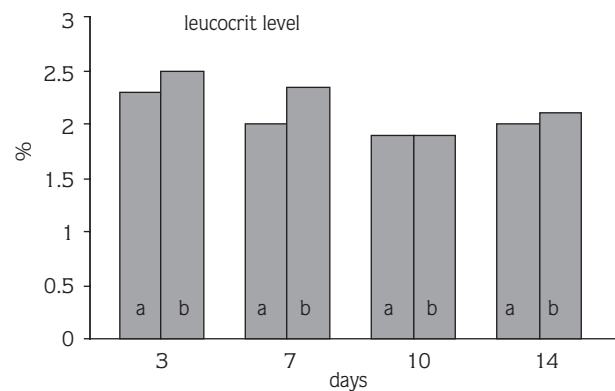


Figure 2. Leucocrit level of rainbow trout 3, 7, 10 and 14 days after the last administration of levamisole. a: Control group, b: Experimental group.

significant ($P < 0.001$) after 14 days of treatment (Table 2, Figure 6). Rainbow trout MPO content was affected by levamisole. The blood leucocyte MPO content was increased significantly when rainbow trout were injected

with levamisole for all the assayed times. In myeloperoxidase activity, the maximum numbers of observed cells were 161 and 97 for the experimental and control groups, respectively (Table 2, Figure 4). In the experimental group, total Ig levels 3, 7, 10 and 14 days after the injection are given in Figure 7. Total Ig level in the levamisole-injected group was not significantly different ($P > 0.05$) than that in the control group (Table 2, Figure 7). Lysozyme activity on days 3 and 7 in the experimental group was similar. There was a significant increase in serum lysozyme activity ($P < 0.001$), compared to the control group. On days 10 and 14, lysozyme activity in the serum had declined, but significantly increased in comparison to control values ($P < 0.01$, $P < 0.001$). In lysozyme activity, the maximum level was $1.50 \pm 0.12 \text{ ml mg}^{-1}$ (day 7), and the minimum level was $1.03 \pm 0.04 \text{ ml mg}^{-1}$ (day 14) in the experimental group (Figure 5). In the experimental

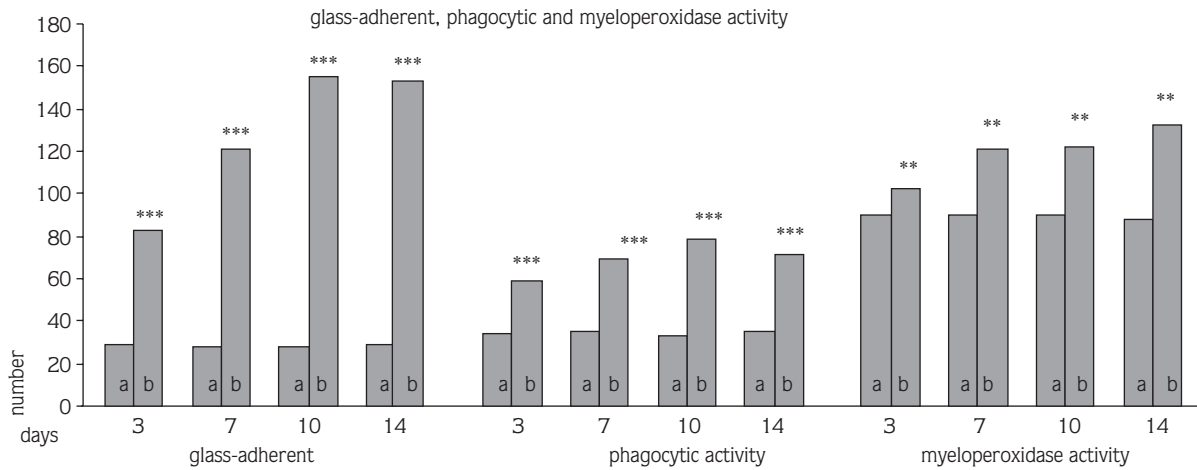


Figure 4. The glass – adherent, phagocytic and MPO activity of blood leucocytes isolated from rainbow trout injected with levamisole a: Control group, b: Experimental group. ** P < 0.01 *** P < 0.001.

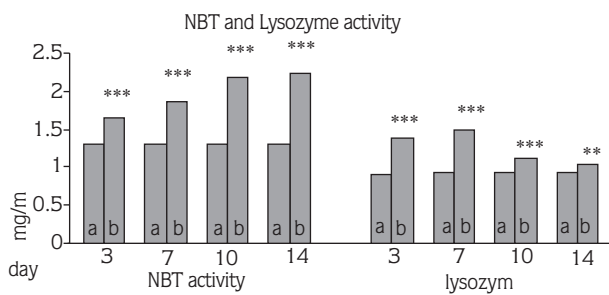


Figure 5. NBT – positive blood neutrophils and lysozyme concentration of serum from rainbow trout injected with levamisole. a: Control group, b: Experimental group. ** P < 0.01 *** P < 0.001.

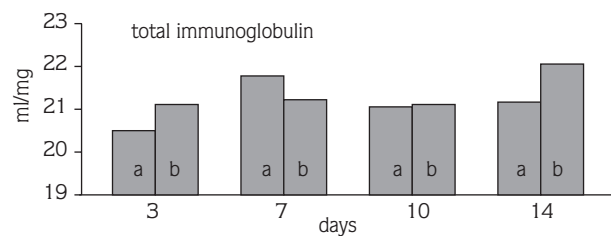


Figure 7. Total Ig level of serum from rainbow trout injected with levamisole. a: Control group, b: Experimental group.

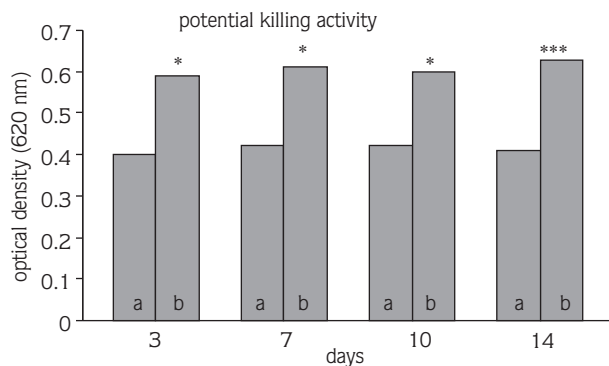


Figure 6. Potential killing activities of neutrophil and monocytes isolated from rainbow trout injected with levamisole. a: Control group, b: Experimental group. * P < 0.05 *** P < 0.001.

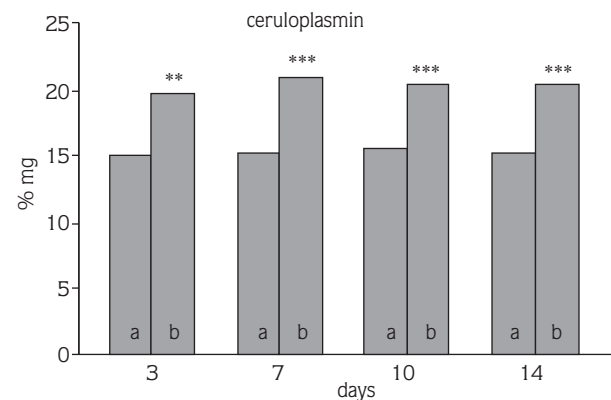


Figure 8. Cp level in serum of rainbow trout injected with levamisole. a: Control group, b: Experimental group. ** P < 0.01 *** P < 0.001.

group, the Cp activity initially increased on day 3 after the injection and reached a maximum after 10 days. Mean Cp values are given in Table 2 and Figure 8.

Discussion

Immunostimulants are commonly used in fish culture. Previous studies have shown the immunostimulant effects of levamisole on a number of fish species. Siwicki (8,9) reported the immunostimulatory activity of levamisole in carp displaying elevated leucocyte and neutrophil numbers, enhancement of phagocytic activity, leucocyte migration and myeloperoxidase activity, increase in lysozyme levels and natural antibody titers. Kajita et al. (20) detected stimulated phagocytic activity, chemiluminescence response and natural killer cell activity in rainbow trout using levamisole. The immunostimulatory effect of levamisole was also shown on the sea bass (15) and on the gilt head seabream (16). In this study, the effects of levamisole, injected intraperitoneally, on the specific and non-specific defense mechanisms of rainbow trout were determined.

Levels of hematocrit and leucocrit are general indicators for fish disease. These parameters give indications about fish health and help to describe abnormalities caused by immunostimulants. Siwicki and Anderson (19) demonstrated that levamisole has less effect on hematocrit, even somewhat variable under high temperature, but has a greater effect on leucocrit level. In this study, there were no significant differences in the hematocrit or leucocrit levels of the control or levamisole-injected fish. Thus, we can speculate that levamisole does not affect the hematocrit or leucocrit levels of fish.

Studies on levamisole reported a significant decrease in leucocyte levels. Anderson and Jeney (21) investigated the effects of some immunostimulants, including levamisole, against *Aeromonas salmonicida* and found that leucocyte levels were increased with immunostimulant treatment except for with levamisole. Siwicki (8) reported that a high concentration of levamisole had a negative effect but the optimal concentration had a positive effect on leucocyte levels in carp. The results in this study are in agreement with the results in previous investigations.

After immunization, in order to measure neutrophil activation in fish the glass-adherent NBT staining method is still widely used. In fact, neutrophil population gives an

important clue about the immune system of fish (22). In previous studies, an increase was detected in glass-adherent NBT positive cell activities 3 days after a levamisole injection (8,9,13,22). Our results for this parameter showed similarity to the results in previous studies. The NBT test is 1 of 2 tests used to determine intracellular killing activity (23). According to the results obtained from the NBT spectrophotometer test in this study oxidative radical production increased at the beginning of the experiment and maintained its activity until the end of the experiment. Siwicki et al. (2) have also observed an increase in oxidative radical production in immunostimulated rainbow trout with the NBT test.

Granulocytes, mononuclear phagocytes or macrophages have an important role in non-specific defense in fish (24). In the present study, phagocytized peripheral blood leucocytes, potential killing activity of neutrophil and monocytes increased in levamisole-injected fish. Similar findings have also been reported by Anderson and Jeney (21), Kajita et al. (25), Sakai et al. (26), Sakai et al. (7) and Siwicki et al. (2).

Siwicki (8) injected different concentrations of levamisole into carp and found an increase in the MPO activities of neutrophil. In the present study, there was a considerable increase in MPO resistance 7 days after the levamisole injection and the increase continued until the end of the experiment. Antibody is an important member of specific defense mechanisms in fish. Total immunoglobulin is a good indicator for determining activation of immunostimulants of abnormalities caused by immunostimulants (2). It was reported that levamisole causes an increase in cellular immunity but it has no effect on antibody production (22). The result in this study that levamisole did not affect specific defense mechanisms supports previous findings. Lysozyme is an important hydrolytic enzyme with a protein character in the non-specific defense system. Lysozyme is found in the phagocytic cells, mucus and serum of several fish species (27). Until the last decade, there was little information on the lysozyme activity of fish (28). The increase in serum lysozyme activity observed in this study shows the immunostimulatory effects of levamisole. Previous studies have also reported immunostimulatory effects of immunostimulants on the lysozyme activity of fish (26,29).

Ceruloplasmin activity is high in infected fish with any kind of disease. Ceruloplasmin activity is regarded as an

indicator of disease in humans, animals and fish (30). There is little information on the ceruloplasmin activity in fish plasma and the effects of immunostimulants on it. In this study, findings on ceruloplasmin activity in the control group show similarity to the results found by Siwicki and Studnicka (30) for carp, but ceruloplasmin activity in the experimental group was high.

The present study demonstrates that a specific concentration of levamisole has a positive effect on the

non-specific defense mechanisms of rainbow trout. Thus, levamisole could be used to prevent mortalities caused by pathogens in fisheries.

Acknowledgments

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