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RESEARCH ARTICLE

Immunostimulating Effect of Levamisole on Spleen and Head-Kidney Leucocytes of Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792)

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Summary

An *in vitro* cultivation method for spleen and head kidney organ section were used to investigate the effects of levamisole on the immune response. After 10 days of culture with 10 µg/ml, 50 µg/ml and 100 µg/ml or no levamisole in the culture media, the non specific defense reaction were measured by glass-adherent NBT (nitroblue tetrazolium) positive cells activation and phagocytic activity. Elevation in phagocytic activity was found for 10 µg/ml and 50 µg/ml levels of levamisole ($P<0.001$), but no differences were found at levels of phagocytic activity in between 100 µg/ml levamisole groups and control groups ($P>0.05$). In the spleen and head kidney leucocytes, increased response to levamisole was shawn for all doses in the glass-adherent NBT positive cells activation.

Keywords: *Levamisole, Immunomodulation, Rainbow trout, Immunity, Head-kidney, Spleen*

Levamisolün Gökkuşağı Alabalığının (*Oncorhynchus mykiss*, Walbaum 1792) Dalak ve Ön Böbrek Lökositlerine İmmunostimulatik Etkisinin Araştırılması

Özet

Bu çalışmada; levamisolün farklı dozlarının gökkuşağı alabalıklarının *(Oncorhynchus mykiss)* dalak ve ön böbrek lökositlerine etkisi *in vitro* olarak araştırılmıştır. Bu amaçla, levamisolün 10 µg ml^{-1}, 50 µg ml^{-1} ve 100 µg ml^{-1} dozunun uygulandığı bir kültür ortamında 15ºC'de 10 günlük dalak ve ön böbrek hücre kültürleri yapıldı. Lökosit düzeyi, glass-adherent NBT pozitif hücre aktivasyonu ve fagositik aktivite gibi nötrofillerin metabolitik aktivitelerinin ölçülmesi ile nonspesifik savunmadaki değişim ortaya konulmuştur. 10 µg ml^{-1} ve 50 µg ml^{-1} levamisol dozlarının uygulandığı deneme gruplarında bir artış gözlendiği ($P<0.05$) halde 100 µg ml^{-1} dozunun uygulandığı grupta hem dalak ve hem de ön böbrek preparasyonlarında kontrol grubuna oranla istatistikî olarak bir farklılık tespit edilemedi ($P>0.05$).

Anahtar sözcükler: *Levamisol, İmmunomodulatör, Gökkuşağı Alabalığı, Bağışıklık, Ön böbrek, Dalak*

INTRODUCTION

Non-specific mechanisms are important in the defence of all multicellular animals against pathogenic micro-organisms. In the cellular part of the non-specific defence, mononuclear phagocytes or macrophages play a central role. They are distributed in most tissues and organs of body and participate in several physiological and pathological processes. These cells can change their function and morphological characteristics, depending on the nature of signals they receive [1]. Because of the risk of developing environmental drug resistance, it is important to minimize the use of anti-bacterial drugs in fish rearing [2]. Levamisole and other immunostimulants are a new alternative in this area. It is possible to meet studies on determination of stimulatory effect of immunostimulants

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or fish phagocytes *in vitro* and *in vivo* [2-10].

Levamisole has been extensively used in both human and veterinary medicine as an anti-helmintic agent [11]. This agent is able to enhance the response of mammalian T-lymphocytes and macrophages [12]. In vitro studies showed that levamisole enhanced phagocytosis, respiratory burst, lymphocyte proliferation and plaque forming cells activities in fish [13,14]. Effects of levamisole on immune system of *Cyprinus carpio*, *Oncorhynchus mykiss*, *O. kisutch* and *Sparus aurata* have also been determined by *in vivo* studies [5,15-21]. Although levamisole is a registered and accepted drug for the European community and the United States Food and Drug Administration, its metabolism is not well known. Toxicity and tissue residues have been reported after its use in some animals but not for fish [22]. It is important to measure changes in specific and non-specific immune system of fish that are exposed to immunostimulants, pollutants, pathogen organisms and stress factor. Oxydative and adherent activities of neutrophils can be observed by using nitroblue tetrazolium (NBT) [23]. It is also possible to determine activation levels of blood and other tissue phagocytic cells by using sheep red blood cells (SRBC) [24]. Since *in vivo* experiments are very expensive, researchers prefer *in vitro* studies. Small fish numbers used in experiments, less time period for experimental study, easy control of fish feeding and water temperature are advantage of *in vitro* experiments. Therefore, *in vitro* studies consist of bacteria and immunostimulants have been reached great number in last decade. In this study, it was aimed to detect effects of levamisole on immune system of rainbow trout by *in vitro* study.

MATERIAL and METHODS

Fish

Non-vaccinated Rainbow trout weighing 200-250 g obtained from a local fish farm were kept in 400 lt recirculated tanks and the fish fed a standard diet until use.

In vitro Assay and Immunisation

Fish used for *in vitro* assay were anaesthetized with benzocaine (Sigma) and body surface cleaned with 70% alcohol. After insizion, spleen and anterior kidney were removed and transferred to sterile petri dishes. They were cut to about 5 mm pieces. Then, pieces were transferred to 3 petri dishes containing 2% foetal calf serum, 100 IU ml^{-1} penicilin, 100 mg ml^{-1} streptomycin, 7 ml Essential Minimal Eagle's Medium (EMEM) containing 10 IU heparin and 10 $\mu g\ ml^{-1}$, 50 $\mu g\ ml^{-1}$, 100 $\mu g\ ml^{-1}$ levamisole respectively.

Tissue pieces of control group were transferred into medium containing 0.1 ml phosphate buffered saline (PBS) (pH: 7.2). Incubation continued for 10 days at 15°C temperature and half of medium was changed daily. At the end of this period, tissue pieces were transferred into a petri dish containing 7 ml EMEM and squeezed in a nylon netting for mixing cells into medium, then centrifuged at 300xg for 10 min. Cells were re-suspended in 1 ml EMEM and used for assay. In order to determine glass-adherent NBT (+) cell activation and phagocytic activity, spleen and head-kidney of all fish were used in the study. Three replicates were carried out for each test.

Immunological Assay

Glass-adherent NBT (+) cells activation of neutrophils was conducted by using NBT stain as described in Anderson et al.[23].

Statistical Analysis

Statistical analysis of results was made using the Minitab Statistical Software Release 10. Student's t-test was performed to determine the statistical significance of mean values compared to control values. Differences were considered statistically significant when $P<0.05$.

RESULTS

In this study, head-kidney and spleen tissues of Rainbow trout were exposed to various concentrations of levamisole and results on non-specific immune system are given on *Fig. 1* and *Fig. 2*.

Glass-adherent NBT (+) cells activation of spleen and head-kidney leukocytes of Rainbow trout exposed to levamisole was found to be higher than un-treated control group.

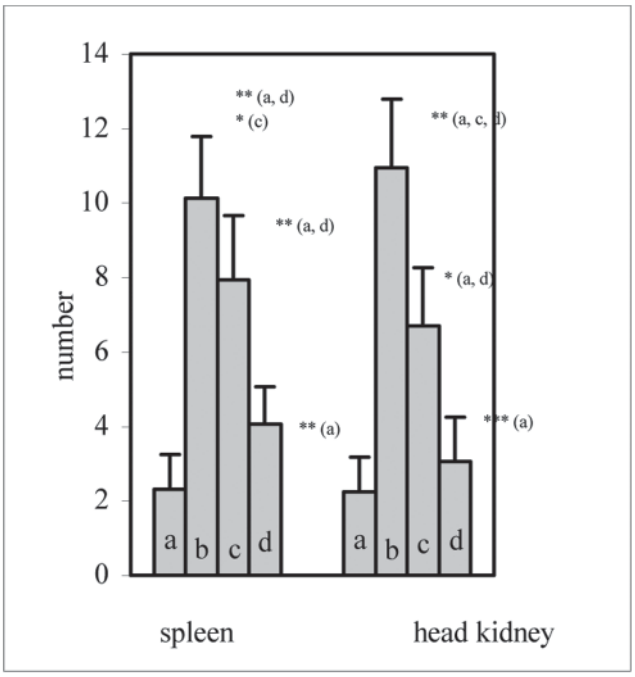
The cells usually appear to have a characteristic blue hole surrounding the otherwise pale or opaque cytoplasm. The specimens exposed to 10 $\mu g\ ml^{-1}$ and 50 $\mu g\ ml^{-1}$ had more neutrophils in their spleen and head-kidney compared to those unimmunized control fish. However, the spleen exposed to 100 $\mu g\ ml^{-1}$ levamisole showed higher activation than that of head-kidney samples. In terms of NBT positive cells activation in spleen samples, 13, 11 and 6 cells were counted in each area according to dosage respectively, but this number was only 6 cells for control. Head-kidney samples, these numbers were 14, 9 and 6 cells for experimental groups and 4 cells the control group.

In spleen and head-kidney samples incubated in low doses of levamisole, 10 $\mu g\ ml^{-1}$ and 50 $\mu g\ ml^{-1}$, phagocytic activity increased compared to control system. Phagocytic activity in spleen was found to be higher than control and it was lower in head-kidney but they were not statistically significant ($P>0.05$).

There was statistically significant differences between spleen and head-kidney samples in terms of phagocytic activity ($P<0.05$), but higher phagocytosis occurred in

spleen exposed to 10 µg ml^{-1} levamisole with phagocytosis of 63 SRBC.

Phagocytic activity was observed as 47 and 25 number in other experimental group respectively but this number was 27 for control group. Phagocytic activity in head-kidney leukocytes were 54, 41 and 31 for experimental groups and 31 for control group.



* P<0.01, ** P<0.001, ***P<0.05; **a:** Control, **b:** 10 µg/ml levamisol, **c:** 50 µg/ml levamisol, d: 100 µg/ml levamisol

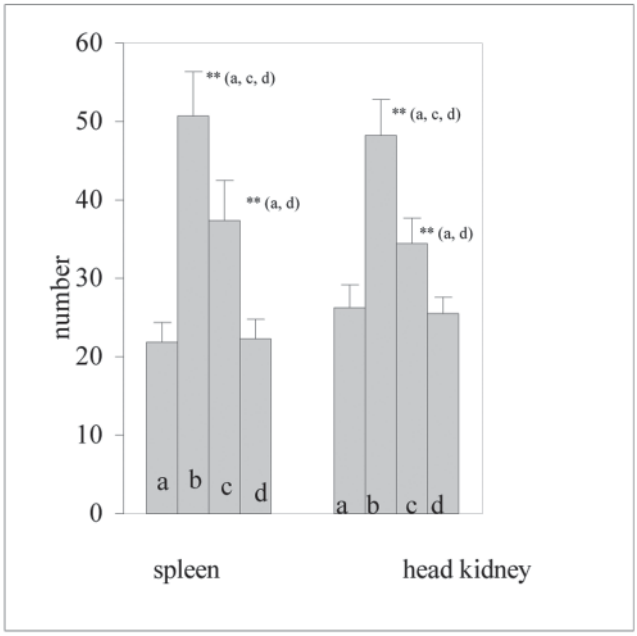
Fig 1. Glass-adherent NBT positive cells activation of head-kidney and spleen of Rainbow trout incubated with levamisole

Şekil 1. Levamisol ile inkübe edilen gökkuşağı alabalığının dalak ve ön böbrekteki glass-adherent NBT pozitif hücre aktivasyonu

DISCUSSION

Fish losses due to infectious diseases has been increased as a consequence in recent years as studies on fish immunology became more popular. Nowadays immunostimulants are used against infectious agents in fisheries. Immunostimulants have a stimulatic effect especially on non-specific defence mechanism of fish and inhibits pathogenic agents. Immunostimulatory effects of levamisole are observed in vertebrates. It has been widely shown there is an that increase of phagocytosis in macrophage and polymorphonuclear cells [25] cytokine secretion of macrophages [26] and in antibody resistance with immunisation [27].

Phagocytosis is an important mechanisms of non-specific immune resistance against pathogenic factors and host defence. Studies demonstrated an increase in phagocytic activity, when given as a bath treatment of



* P<0.01, ** P<0.001, ***P<0.05; **a:** Control, **b:** 10 µg/ml levamisol, **c:** 50 µg/ml levamisol, d: 100 µg/ml levamisol

Fig 2. Phagocytic activation of leukocytes of spleen and head-kidney of Rainbow trout incubated with levamisole

Şekil 2. Levamisol ile inkübe edilen gökkuşağı alabalığının ön böbrek ve dalaktaki lökositlerin fagositik aktivasyonu

levamisole to Rainbow trout [28], injection [15,18], in vitro [14] and orally to gilthead seabream [7]. In this study, results showed similarity with previous studies. Mulero et al.[29] revealed that fish phagocytes are not target cells for levamisole after observing no increase in migration, phagocytic or bactericidal activity after incubation of head-kidney phagocytes of gilthead seabream with levamisole. In this study, although phagocytic and bactericid activity in head-kidney cell preparation were found to be different for 10 µg ml^{-1} and 50 µg ml^{-1} levamisole concentration than control group (P<0.001), it was not significantly different at 100 mg ml^{-} concentration. Levamisole increase the production of macrophage activiting factor (MAF) by spleen lymphocytes and this is a sign that MAF has activation effects on phagocytes. Another evidence, studies carried out on human, increase of interleukin with levamisole application shows its effect on macrophages [30].

In vitro and in vivo studies carried out with other immunostimulants showed an increase in phagocytic cells. Stimulation of head-kidney macrophages in atlantic salmon [2,9], neutrophils in catfish [3] and spleen and kidney leucocytes in revealed that different glucan derivatives have influence on phagocytic activity.

Results in Jeney and Anderson [28] studied determination of phagocytic proportion by using SRBC after spleen incubation of rainbow trout with levamisole show similarity with our results in terms of activation although spleen cells suspension was higher.

Glass adherent NBT positive cell activation of peripheral blood neutrophils is an important non-specific defence mechanism of fish. Measurement of this activation gives clue about fish health. It has been reported that glass adherent NBT positive cells activation in circulation blood of Rainbow trout [15,18,28] and *Cyprinus carpio* [20,21] was greatly increased by levamisole application. In this study, glass adherent NBT positive cell activation in spleen and head-kidney cell preparation was also higher for all levamisole doses.

In addition, cell activation was higher in spleen than head-kidney in all doses except the 10 mg ml^{-1} dose. It is known that high levamisole doses cause immunosuppression. In our study, even the 100 µg ml^{-1} levamisole dose did not cause suppression in spleen and head-kidney preparation. In vivo and in vitro studies have also shown that high levamisole doses could not stimulate immune mechanisms activation [14,29]. But the effective mechanism has not been explained yet.

Results in this study showed stimulating effect of levamisole on spleen and head-kidney leukocytes. Using immunostimulant alone or with adjuvants against pathogens in fisheries make immunostimulants more important. In conclusion, by using levamisole or other immunostimulants in fish culture units economical losses due to pathogens may decrease and a healthy fish culture can be maintained.

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