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HİJRAN YAVUZCAN YILDIZ

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Research Article

Plasma Lysozyme Levels and Secondary Stress Response in Rainbow Trout, *Oncorhynchus mykiss* (Walbaum) after Exposure to Leteux-Meyer Mixture

Hijran YAVUZCAN YILDIZ*

Department of Fisheries and Aquaculture, Faculty of Agriculture, Ankara University, 06110 Ankara - TURKEY

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Abstract: Leteux-Meyer mixture (LMM) contains formalin and malachite green and can be externally applied to fish as an antimicrobial agent. To determine the effects of prophylactic treatment with LMM on plasma lysozyme levels and the secondary stress indices, alterations in plasma glucose, chloride, sodium, potassium and hematocrit were monitored in healthy rainbow trout, *Oncorhynchus mykiss* W. Fish were i) exposed to 25 ppm Leteux-Meyer mixture for 60 min and ii) the same treatment followed by 60 min recovery in clean freshwater. Treatment with LMM resulted in a decrease in plasma lysozyme values. After a recovery period of 60 min plasma lysozyme levels were not restored to control values. Treatment of fish with LMM elicited marked elevations in plasma glucose and plasma potassium values. Plasma chloride and sodium values dropped after LMM treatment. Recovery for 60 min was not sufficient to restore the electrolyte levels. Hematocrit values were not affected by LMM treatment.

Key Words: *Oncorhynchus mykiss*, rainbow trout, Leteux-Meyer mixture, secondary stress, plasma lysozyme

Leteux-Meyer Karışımına Maruz Bırakılan Alabalıklarda, *Oncorhynchus mykiss* (Walbaum) Plazma Lizozim Seviyesi ve Sekonder Stres Yanıt

Özet: Leteux-Meyer karışımı (LMK), formalin ve malahit yeşilinden oluşan, balıklara eksternal olarak uygulanabilen antimikrobiyel bir karışımdır. Profilaktik amaçlı LMK uygulamanın, plazma lizozim seviyesi ile sekonder stres indeksine etkileri, sağlıklı gökkuşağı alabalıklarında plazma glukoz, klorür, sodyum, potasyum ve hematokrit değerlerindeki değişimler izlenerek gözlemlenmiştir. Balıklar i) 60 dakika süre ile 25 ppm LMK çözeltisine maruz bırakılmıştır ve daha sonra ii) aynı uygulamayı takiben 60 dakika temiz suda tutulmuşlardır (iyileşme periyodu). LMK uygulanan balıklarda plazma lizozim seviyesi düşmüştür. Temiz suya dönen balıklarda (iyileşme periyodu) plazma lizozim seviyesi kontrol değerlerine dönmemiştir. LMK uygulaması plazma glukoz ve potasyum değerlerinde belirgin bir artışa yol açmıştır. Plazma sodyum ve klorür değerleri LMK uygulamasını takiben düşmüştür. 60 dakikalık iyileşme periyodu elektrolitlerin seviyelerinin normale dönmesi için yeterli olmamıştır. Hematokrit değerleri LMK uygulamasından etkilenmemiştir.

Anahtar Sözcükler: *Oncorhynchus mykiss*, gökkuşağı alabalığı, Leteux-Meyer karışımı, sekonder stres, plazma lizozim

Introduction

An emerging husbandry practice in fish farming is the prophylactic use of aquaculture chemicals to reduce the incidence of topical bacterial, protozoal and fungal infections (1). It is known that prophylactic treatments may be stressors in reared fish. Stressors affect fish physiology and behavior, which may be reflected by immunosuppression (2). Combined treatment with malachite green and formalin (Leteux-Meyer mixture (LMM) has been extensively used in aquaculture for the treatment or prevention of fungal and parasitic infections (3). The effects of formalin and malachite green on blood characteristics have been reported for different fish species (4-10). The synergistic effect of the components of LMM for the treatment of *Ichthyophthirius multifiliis* was demonstrated first by Leteux and Meyer (11). LMM is also effective against infections with *Costia, Trichodina, Chilodonella, Scyphidia* and *Trichophyra* (12).

*E-mail. Hijran.Yavuzcan@agri.ankara.edu.tr

The possible effects of externally applied antimicrobial agents on the innate components of immunity have received little attention. No studies have addressed the interactions between LMM, lysozyme activity and the stress response. In fish, lysozyme, an enzyme with antibiotic properties that is released by leucocytes, has a broader spectrum of activity than mammalian lysozyme (13), and lysozyme has been frequently used as an indicator of non-specific immune functions (14). It is able to cause damage to the bacterial cell walls (15). Stressors can induce variations in lysozyme activity (16).

The aim of the present study was to determine whether exposure to LMM could change plasma lysozyme activity. The stress response of the fish was evaluated by measuring plasma glucose, sodium, potassium, chloride and hematocrit.

Materials and methods

Experimental fish

Sixty clinically healthy rainbow trout (*Oncorhynchus mykiss*) with a mean body weight of 190 ± 10 g were obtained from a commercial farm, located at Kesikköprü Dam Lake, Central Anatolia. Experiments were carried out at the Department of Fisheries and Aquaculture, Ankara University. Before the experiment fish were maintained in 200-l fiberglass tanks containing static freshwater. Experiments were performed at a water temperature of 12 ± 2 °C.

Experimental procedure

LMM was formulated as a concentrate by dissolving 3.3 g of malachite green in 1 l of formalin (37%).

In the LMM exposure (N = 15) and recovery tests (N = 15), fish were exposed to: i) 25 ppm LMM for 60 min, and ii) 25 ppm LMM for 60 min followed by 60 min recovery in freshwater. The fish were sampled immediately after exposure or after exposure and recovery. Control fish (30 in total) were sampled at the same intervals. Fish were removed from the test tanks by hand net. Fish were not anesthetized before cardiac puncture. Control fish were handled in the same way.

Blood/plasma collection

Blood samples were drawn by cardiac puncture into heparinized syringes and then centrifuged in tubes. Plasma was separated and stored at –18 °C until analyzed for lysozyme. Other parameters were immediately measured after blood sampling.

Analytical procedures

Plasma lysozyme was analyzed with the turbidometric assay (17). Plasma (100 µl) was added to 1900 µl of *Micrococcus lysodeikticus* aqueous suspension (0.2 mg mL^{-1} in 0.05 - H_2PO_4). Two readings of adsorption were carried out at 530-nm wavelength with a spectrophotometer (Shimadzu UV 2100) after 30 s of mixing and after 4.5 min of mixing. The unit of lysozyme activity (U) was defined as the amount of enzyme that caused a decrease in adsorbance of 0.001 min L^{-1}.

Blood glucose level was determined by GOD-PAP method with the kit prepared by Linear Chemicals (Barcelona, Spain). Plasma sodium and potassium levels were determined by a colorimetric method using the kits of Teco Diagnostics (California, USA). Plasma chloride was assessed by Mercuric Thiocyanate Direct Method with the kit prepared by Linear Chemicals.

Hematocrit was measured immediately by drawing blood samples into heparinized capillary tubes, which were centrifuged at 12,500 rpm for 4 min (18).

Statistical analysis

All data were statistically analyzed by one-way analysis of variance followed by Duncan's multiple-range test where appropriate.

Results

Treatment with LMM for 60 min resulted in a decrease in plasma lysozyme values ($P < 0.05$). After recovery for 60 min, plasma lysozyme levels were not restored to control values (Table 1). Mean values of secondary stress parameters (plasma glucose, chloride, sodium and potassium and hematocrit) of rainbow trout exposed to LMM for 60 min and 60 min of recovery are presented in Table 2. Fish exposed to LMM had higher plasma glucose values than did the control fish ($P < 0.05$). Plasma glucose values did not return to control values after recovery of 60 min. The changes in the values of plasma chloride, sodium and potassium were statistically significant ($P < 0.05$). Plasma chloride

Table 1. Plasma lysozyme values of rainbow trout i) after treatment with Leteux-Meyer mixture (LMM) for 60 min and ii) after treatment with LMM for 60 min followed by 60 min in freshwater (recovery).

	LMM exposure period 60 min	Recovery period 60 min
Plasma lysozyme (10^3U/ml)		
Treatment	$0.40 \pm 0.10^{a*}$B	0.37 ± 0.07^{a}B
Control	1.33 ± 0.19^{a} A	1.16 ± 0.15^{a} A

* Different superscripts in a row refer to significant differences between the treatment and recovery groups ($P < 0.05$).
** Different capital letters in a column refer to significant differences between the controls and the treatment groups ($P < 0.05$).

Table 2. Means ± SE for secondary stress indices in rainbow trout i) after treatment with LMM exposure for 60 min and ii) after treatment with LMM for 60 min followed by 60 min of recovery in freshwater.

Secondary Stress Parameters	LMM exposure period 60 min	Recovery period 60 min
Glucose (mg dL^{-1})		
Treatment	114.74 ± 1.84^{a}*A	117.12 ± 0.35^{a}A
Control	93.84 ± 0.36B**	92.04 ± 0.25B
Chloride (mEqL^{-1})		
Treatment	132.73 ± 0.16^{b}B	165.62 ± 0.52^{a}A
Control	144.22 ± 0.14A	140.10 ± 1.22B
Sodium (mEqL^{-1})		
Treatment	96 ± 2.90^{a}B	88 ± 0.18^{b}B
Control	156 ± 2.50A	151 ± 1.50A
Potassium (mEqL^{-1})		
Treatment	6.58 ± 0.11^{a}A	6.35 ± 0.18^{a}A
Control	4.90 ± 0.20B	4.80 ± 0.99B
Hematocrit (%)		
Treatment	34.00 ± 0.01^{a}A	33.62 ± 0.10^{a}A
Control	34.44 ± 0.24A	33.00 ± 0.16A

*Different superscripts in a row refer to significant differences between the treatment and recovery groups ($P < 0.05$); no significant differences were found between the control groups.
** Different capital letters in a column refer to significant differences between the controls and the treatment groups ($P < 0.05$).

declined following LMM exposure, but increased during the recovery period. Plasma sodium, in general, decreased in fish treated with LMM and during the recovery. After 60-min exposure to LMM and in the recovery period potassium values increased when compared to the control. No differences in hematocrit values were found between the groups ($P > 0.05$).

Discussion

LMM exposure for 60 min in rainbow trout was associated with marked reductions in plasma lysozyme activity. The decline in activity persisted after a recovery period of 60 min. In contrast, Demers and Bayne (13) reported that, following exposure to a handling stressor, lysozyme activity was significantly increased. Lysozyme

activity could be dependent on the degree of stress intensity and its duration (16) and type of stressors (19). The lack of consistency in the lysozyme activity suggests that the influence of stressors in lysozyme remains controversial. It is possible that the relationship between stress and immune response is nonstable (20).

Plasma glucose has been considered a very sensitive stress parameter in detecting sublethal stress responses (21), relating to cortisol-mediated gluconeogenesis (22,23). The LMM treatment in the present study had a stimulating effect on plasma glucose, and its levels did not return to control values after 60-min recovery. A rapid rise in the glucose concentration (hyperglycemia) has been shown in the blood of fish after exposure to different stressors (2,8,23). In the present study the recovery period of 60 min was not sufficient to restore the glucose levels. Plasma chloride decreased after exposure to LMM, but the values for treated fish after recovery period for 60 min exceeded the control values, which is hard to explain. Plasma sodium values decreased in all LMM-treated fish. A substantial depression in plasma chloride and sodium levels in stressed salmonids has been widely reported (24). In the present study, the plasma potassium values increased after LMM exposure for 60 min and a recovery period of 60 min. Exposure of the tilapia (*Oreochromis niloticus*) to a mixture of formalin, malachite green and methylene blue resulted in unsteady changes in plasma potassium values (8). In the present study, hematocrit values tended to stay within the normal range, in agreement with findings on juvenile rainbow trout treated with chloramine-T (25). Conversely, it has been reported that hematocrit values increased after stressful stimuli (7,8,26,27). The peculiarity of alterations in hematocrit may depend on the duration of exposure as well as the concentration of toxic chemicals, as stated by Vosyliene and Kazlauskiene (27).

In conclusion, variation in the blood chemistry parameters and in particular the elevated plasma glucose levels of rainbow trout treated with LMM can be interpreted as reflecting a stress response. Hence, the use of LMM for prophylactic treatment causes stress. A period of 60 min was not sufficient for recovery from LMM exposure. LMM treatment induced a decrease in lysozyme. Various hormonal pathways but also severe stressors have been considered responsible for lower lysozyme activity in fish during stress (16,20).

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