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Research Article

The Effect of Oxytetracycline on Non-Specific Immune Response in Sea Bream (*Sparus aurata* L. 1758)

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Abstract: The aim of the present study was to evaluate the immunomodulatory effects of orally administered oxytetracycline HCl (OTC) of 75 mg/kg body weight for 10 days on the immune response in sea bream (*Sparus aurata* L. 1758) using the nitro blue tetrazolium (NBT) reduction test and erythrocyte and leucocyte counts. Administration of 75 mg/kg OTC for 10 days enhanced the non-specific immune response, and increased NBT (+) cell, total erythrocyte and leucocyte numbers in sea bream at 23 ± 1 ºC. The effect of OTC on the immune system lasted around 21 days after ceasing the administration and the parameters evaluated then returned to normal levels.

Key Words: Neutrophil, nitroblue tetrazolium, oxytetracycline, sea bream

Çipura Balığı (*Sparus aurata* L. 1758)'nda Oksitetrasiklinin Spesifik Olmayan İmmun Cevap Üzerine Etkisi

Özet: Bu çalışmanın amacı, 10 gün boyunca 75 mg/kg canlı ağırlık dozda oral yolla verilen oksitetrasiklin HCl (OTC)'nin çipura (*Sparus aurata* L. 1758) balıklarının immun sistemine etkisinin nitro blue tetrazolium (NBT) testi, eritrosit ve lökosit sayımı ile araştırılmasıdır. Çipura balığına 23 ± 1 ºC'de 10 gün süreyle uygulanan 75 mg/kg OTC tedavisi spesifik olmayan immun cevabı geliştirdi, NBT (+) hücreleri, total eritrosit ve lökosit sayısını arttırdı. OTC'nin bağışıklık sistemine etkisi ilaç uygulamasının bitiminden yaklaşık 21 gün sonraya kadar sürdü ve incelenen parametreler normal değerlerine ulaştı.

Anahtar Sözcükler: Nötrofil, nitroblue tetrazolium, oksitetrasiklin, çipura

Introduction

Oxytetracycline (OTC, Sigma) is a bacteriostatic compound with a broad antibacterial activity against both Gram-negative and Gram-positive microorganisms, and both aerobic and anaerobic species (1). OTC has been approved for use in aquaculture by the US Federal Drug Administration (2) and is also used very widely in Turkey. It is a broad-spectrum antibiotic widely used for the treatment of systemic bacterial infections in cultured finfish (3,4).

OTC is recommended for therapy or prophylaxis for diseases like columnaris disease, edwardsiellosis, and enteric red-mouth disease (3,5). Depending on the infection, fish species and temperature, OTC oral dosage regimes range from 50 to 300 mg/kg body weight per day for 3-14 days (4). Under field conditions the daily recommended OTC dosage is 50-75 mg/kg body weight for 10 days (5).

Antibiotics may inhibit growth or even kill the bacterium depending on the mode of action and concentration. It has been recently reported that OTC and other, similar compounds may interfere with normal immunological processes in fish, birds and mammals (6) and may decrease the immune response of the animal to infective agents.

Interactions between antibiotics and the immune mechanisms in fish have been demonstrated in several studies (4,7-10). The immunomodulatory effects of antibacterials and other drugs are numerous and have proved different for different drugs tested. Some drugs have been shown to stimulate the defence mechanisms. For example, levamisole, an antihelmintic drug, exerted a stimulating effect on the non-specific as well as the specific defence mechanisms in rainbow trout (*Oncorhynchus mykiss*), resulting in increased protection against disease when administered (7). In contrast, Grondel et al. (4), Rijkers et al. (8,9), and Siwicki et al. (10) have shown that oxytetracycline suppresses the immune functions in carp (*Cyprinus carpio*) and rainbow trout. Lunden et al. (11) and Siwicki et al. (10) have shown that both oxytetracycline and oxolinic acid suppress antibody production as well as the level of circulating leucocytes in rainbow trout. The phagocytic activity of whole blood leucocytes is suppressed by oxytetracycline but stimulated by oxolinic acid. As can be seen above and as stated by van der Heijden et al. (12) many reports have described either positive, negative or no effect at all of antimicrobial agents on the defence system. Unfortunately the number of studies conducted on fish species is very limited. Thus, the available results are based on few trials carried out with different antibiotics and species. To estimate the immunomodulatory effect of antibiotics, different tests for determining the activity of immunological defence mechanisms must be optimised and standardised (12). The nitroblue tetrazolium (NBT) assay is the most useful test for the rapid determination of active neutrophils (7,12-15).

The aim of the present study was to evaluate the immunomodulatory effects of orally administered OTC HCl on the non-specific immune response in sea bream (*Sparus aurata* L. 1758) using the NBT reduction test and erythrocyte and leucocyte counts.

Materials and Methods

Hatchery grown sea bream (*Sparus aurata*) (mean weight of 180 ± 10 g) were obtained from the Faculty of Fisheries, Ege University, Turkey. The fish were kept in seawater of 36‰ and 23 ± 1 ºC in 500-l fibreglass tanks with flow rates of 7-8 l/s in the fish disease laboratory. The experimental fish were randomly sampled and divided into a test (n = 20) and a control group (n = 12). The test fish were hand-fed steam pellets (Ø = 4 mm, Pınar HP = 46%) medicated with OTC HCl (Sigma) at a predetermined rate (4 pellets/individual fish) every day at 10:00 a.m. The dosage of OTC was 75 mg/kg of body weight. The control group was given the same amount of untreated feed at the same time. Blood samples were taken at weekly intervals starting from day 11. Every sampling 3 fish were used. Prior to sampling the fish were anaesthetised with benzocaine (50 mg/l, Sigma). Around 0.5 ml of blood was taken from each fish heart using a heparinised syringe. Three drops (about 0.05 ml each) of the blood sample were used for the NBT test and another 0.05 ml for the cell count. The sample was diluted (1/50) in 2.45 ml of Leibovitz medium (L-15, Sigma).

NBT (Sigma N-6876) was used to determine the respiratory burst activity by following a modified method (13). Samples from the blood of each fish were dropped immediately on a glass coverslip that was placed on a moist paper towel in 60 mm diameter petri dishes and incubated for 30 min. Thus neutrophils (including some monocytes and macrophages) were adhered to the glass. The excess cells were washed with phosphate buffered saline (PBS) and excess solution was drained off. The glass coverslip was turned upside down onto a drop (0.05 ml) of 0.2% NBT in PBS solution on a glass microscope slide, incubated for 20 min, and then it was observed microscopically (x400 magnification). The cells that took up the blue dye were counted in 3 microscope areas and were compared with untreated control samples.

Leibovitz 15 (Sigma L-4386) with 7.5‰ NaCl (pH 7.4) was used as diluent for the blood cell count after adding 50 µg/ml gentamycine, and 100 IU penicillin to avoid any contamination, and filtering (22 µm membrane filter). The erythrocyte and leucocyte numbers were counted in diluted blood samples (1/50 in L-15) (16). The samples were transferred in a sterile pipette to a Thoma chamber.

For statistical analysis, means and standard deviations for all test values were calculated, and Student's t-test was used to determine differences between the groups ($P < 0.05$).

Results

After 10 days of OTC treatment, the number of NBT (+) cells increased considerably in comparison to the control group. However, after the first sampling, i.e. 7 days after the beginning of sampling, the number of NBT (+) cells in the treated group declined sharply and 21 days later the values levelled off to those of the control group (Figure 1). This result confirms that OTC reduced phagocytic activation of phagocytic cells and it was removed from the body after 21 days of treatment.

At the first sampling, erythrocyte numbers in the OTC treated fish were significantly higher than those in the control fish ($P < 0.05$), but the numbers dropped drastically and levelled off in the 2 groups up to the second sampling (Figure 2).

Leucocyte numbers exhibited quite different patterns: values in the OTC treated fish were considerably higher than those in the control fish in the first sampling, but during the second sampling there was a slight decrease in the treated group and sharp increase in the control group. Towards the third sampling leucocyte numbers declined in both groups and finally the difference disappeared at the end of the trial period (day 21 or fourth sampling) (Figure 3).

Discussion

The rapid intensification in finfish farming has drastically increased the use of antimicrobials, in particular antibiotics. OTC is a broad-spectrum antibiotic used widely for the treatment of systemic bacterial infections in cultured finfish. Interactions between antibiotics and the immune mechanisms in fish have been demonstrated in several studies (4,7-10). Because the antimicrobial drug tested in the present study has widespread therapeutic applications in fish farming, it is important to understand its effects on the immune parameters. Appropriate elimination of bacteria requires effectiveness of the antimicrobial drug as well as a well-functioning defence system in the fish (17).

The drug may have different effects on the fish's immune system depending on the species, water temperature and drug used (12). There are several reports on the effects of different antimicrobial drugs on phagocyte functions in mammals (18,19). The effects reported vary (no effects, suppression or synergy with immune system, i.e. enhancement of immune functions) depending on the drug studied. There is limited information available about the effects of antimicrobial drugs on fish phagocytes.

As emphasised recently by Lunden et al. (19) there are 2 main phagocytes, namely mononuclear phagocytes (tissue macrophages and circulating monocytes) and polymorph nuclear phagocytes (mainly neutrophils). These are multifunctional non-specific cells in the immune system of fish and other vertebrates. Macrophages are considered the main phagocytic cells in fish and they are also the dominant phagocytes in the head kidney. The polymorph nuclear leucocytes, mainly neutrophils, are the main phagocytic cells in blood.

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 that are distinctive on microscopic examination. NBT (+) neutrophils were observed (13,14). These cells were round and multilobar and the granules were stained blue-green.

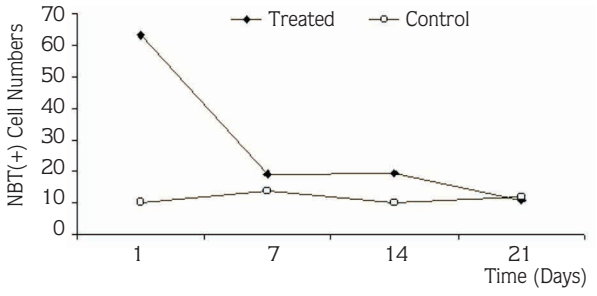

Figure 1. Number of the NBT (+) cells in treated and control groups.

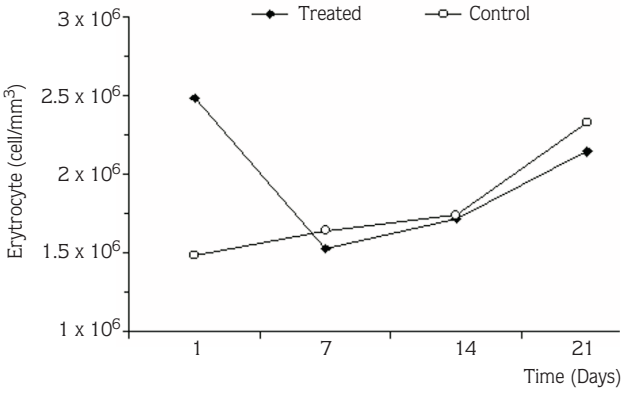

Figure 2. Number of erythrocytes in treated and control groups.

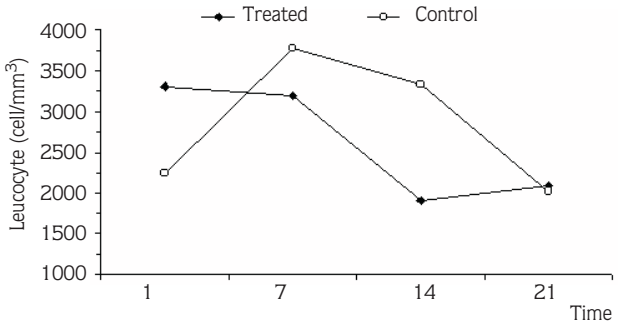

Figure 3. Number of leucocytes in treated and control groups.

During the first week after OTC treatment, the number of NBT (+) cells was very high but declined rapidly until the end of this week (Figure 1). Similarly, erythrocyte numbers in OTC treated fish were significantly higher than those in the control fish in the first sampling, but the numbers levelled off in the 2 groups up to the second sampling. This result confirms that OTC reduced the phagocytic activation of phagocytic cells and it was removed from the body after 21 days of treatment. The effect of OTC on leucocyte numbers was not so clear since the values in the control fish increased in the second sampling (Figure 3), possibly because of a kind of bacteriological infection.

As mentioned before, the effects of antimicrobial drugs vary but are generally suppressive. van der Heidjen et al. (12) reported that OTC increases and suppresses the immune system in common carp and rainbow trout. Similarly, Siwicki et al. (10) reported a suppression of the phagocytosis and NBT activity of rainbow trout spleen phagocytes after i.p. injection of 10 mg/kg of OTC, while Lunden et al. (19) found, in vitro, that widely used drugs (oxolinic acid, oxytetracycline, florfenicol and sulphadiazine in combination with trimethoprim) in most cases suppressed the respiratory burst significantly at the highest drug concentration tested (100 µg/ml) in rainbow trout. Taffaella et al. (20) did not find any effect on respiratory burst activity or phagocytosis of kidney macrophages in turbot after oral administration of oxytetracycline. In contrast, drugs such as levamisole (an anthelmintic drug), exerted a stimulating effect on the non-specific as well as the specific defence mechanisms in rainbow trout (3). In the present study we observed several days after the treatment (75 mg kg^{-1} fish daily over 10 days) that OTC stimulated gilthead sea bream's immune system by increasing NBT (+) cell, total leucocyte and total erythrocyte numbers. This result is in agreement with the findings of Anderson and Jeney (7).

This study clearly showed that the administration of 75 mg/kg OTC for 10 days enhanced the immune response, and increased total erythrocyte and leucocyte numbers in sea bream at 23 ± 1 ºC. The effects of the drug declined considerably during the first week of post-treatment and disappeared completely after 21 days. Since the effects of such drugs may be modified by various factors, such as the mode of administration, dosage, water temperature, size of fish, strain of bacteria and resistance developed, further studies should be carried out under different conditions to give an in-depth knowledge of the immunomodulatory effects of antimicrobials in fish.

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