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Evaluation of dorsal aorta cannulation for immunological studies of grouper (*Epinephelus malabaricus*)

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Blood is often withdrawn to study the immune responses of fish. However, netting, handling and anaesthetising the experimental fish, and drawing blood samples cause severe stress that may alter the effects of immune study protocols and treatments. We evaluated the effect of aorta cannulation, for use in immune studies, on grouper (*Epinephelus malabaricus*) plasma cortisol, total red and white blood cell counts and phagocytosis.

Plasma cortisol increased from 30 to 88 ng/ml 1 day after insertion of the cannula, to a maximum of 951 ng/ml 3 to 5 days after surgery, indicating the groupers were stressed by cannulation and post-cannulation inflammation. Total RBC count decreased, and total WBC count increased after surgery. Following cannulation, the phagocytic index of peripheral blood leukocytes decreased from 100% to 46%. The adverse effects of cannulation were mitigated by continuously immersing groupers in oxytetracycline (OTC), which decreased the recovery period for treated fish. In contrast, OTC-treatment did not markedly improve the recovery of groupers subjected to caudal vessel puncture. Cortisol levels in OTC-treated grouper with caudal vessel puncture were significantly higher than in OTC-treated, cannulated grouper, and remained at a high level until day 13 of the experiment. From day 7 to 13, total RBC and WBC counts in OTC-treated, cannulated groupers were significantly different from those in OTC-treated groupers with caudal vessel puncture. OTC treatment improved the phagocytic index of groupers subjected to caudal vessel puncture, but the phagocytic index was lower than that of groupers subjected to cannulation.

Cannulation minimises visual and handling disturbances, and facilitates standardisation of experimental conditions and quick and easy sampling via the dorsal aorta cannula. Therefore, dorsal aorta cannulation minimises the stress of blood sampling and should prove useful for immune studies in fish.

Key words: cannulation, dorsal aorta, oxytetracycline, hematological analysis, grouper.

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I. Introduction

During the past decade, grouper mariculture has increased throughout Asia. However, many diseases threaten the growth of the grouper industry. Ideally, infections are controlled by immunoprophylactic measures, which pose no danger to the environment and enhance the immune response of fish. Studying the immune responses of fish often entails taking blood samples. No matter how quickly and efficiently they are performed, netting, handling, administering anaesthesia and internal bleeding stress fish. Taking blood samples increases stress substantially, causing marked changes in hormone levels, such as a rapid elevation in plasma cortisol [1–4]. A number of secondary responses occur, including changes in haematocrit values, leukocyte counts, leukocyte phagocytic activity and mitogenic responses [3, 5–7]. As a result, the effects of stress limit the utility of immunology protocols that involve repetitive blood sampling from one or several individuals sharing the same tank.

The use of cannula to sample fish blood, or to introduce substances into fish circulatory systems was first reported nearly four decades ago [8–10]. The method has several distinct advantages. Instead of anaesthetising an animal to collect blood with a needle inserted into a blood vessel or the heart, the insertion of a cannula into a blood vessel allows for repeated sampling or treatment of a conscious animal. Through the cannula it is possible to re-introduce unused erythrocytes to the systemic circulation, substantially reducing the potential for anaemia in an experimental fish. Blood vessel cannulation has been widely adapted for use with air-breathing fish (*Channa argus*) [11], Arctic char (*Salvelinus alpinus* L.) [12], Atlantic salmon (*Salmo salar*) [13, 14], carp (*Cyprinus carpio*) [15, 16], dogfish (*Scyliorhinus canicula*) [17], eels (*Anguilla anguilla*, *A. japonica*, *A. rostrata*) [18–20], flatfish (*Platichthys stellatus*) [21], Japanese flounder (*Paralichthys olivaceus*) [22], rainbow trout (*Salmo gairdneri*) [20, 23–26], striped bass (*Morone saxatilis*) [27], tilapia (*Oreochromis mossambicus*) [28], tuna (*Euthynnus affinis*) [29] and yellowtail [22]. Most of these studies have focused on respiratory, circulatory and digestive physiology, endocrinology [24, 26] or pharmacokinetics [12–14]. Few researchers have used blood vessel cannulation to study biological defense mechanisms in tropical fish.

In this study, we evaluated the effects of dorsal aorta cannulation on grouper to determine whether this technique could be used for immunological studies that require multiple blood samplings, without causing excessive stress to individual animals. Caudal vessel puncture on fish that were similarly bled as the cannulated fish were compared with respect to haematological and phagocytic parameters. Furthermore, the effects of oxytetracycline (OTC) on the recovery of these parameters to normal levels was also studied.

II. Materials and Methods

EXPERIMENTAL FISH

Twenty-four groupers (*Epinephelus malabaricus*; average weight: 605 g; average length: 29·5 cm) were purchased from a local hatchery and acclimated

for 3 weeks in holding tanks containing aerated, filtered, re-circulating sea water. Water salinity was 25 ppt, the temperature ranged from 24 to 26° C and dissolved oxygen was 5·2 to 5·5 ppm. The photoperiod was 12 h light and 12 h dark. Fish were fed commercial feed pellets twice a day, but received no food for 24 h prior to surgery or initial venous puncture. Twelve fish were cannulated, and half of these were continuously immersed in sea water containing 20 ppm oxytetracycline (OTC-treated) until the termination of the experiment to prevent potential bacterial infection [30]. In the control group of 12 fish, half of the fish were also continuously immersed in sea water containing 20 ppm oxytetracycline (OTC-treated). To eliminate pollutants and floating mucus that the fish secreted, about 10 to 20 l of tank water were replaced daily with sea water.

BASELINE BLOOD SAMPLES FROM THE CAUDAL VESSEL

Seven days prior to cannulation, all fish were starved for 24 h and anaesthetised for about 10 min in sea water containing 0·02% benzocaine solution (Sigma, St Louis). One ml of blood samples were taken from the caudal vessel (1 cm below the lateral line) of the control and experimental groups using a 2·5 ml syringe with a 20 gauge needle. Low molecular weight (Sigma) heparin sodium was used as an anticoagulant. It was dissolved in phosphate buffered saline (PBS, 0·01 M) to a final concentration of 14·3 USP/ml and passed through a 0·22 μm filter. The ratio of anticoagulant to blood was 1:9.

CANNULATION OF THE DORSAL AORTA

On the day of cannulation all the fish (which had been starved for 24 h) were anaesthetised in sea water containing 0·1% 2-phenoxyethanol (Showa) until respiratory movements ceased. For cannulation, fish were positioned ventral side up, supported by a soft cloth on a holding rack, and were kept unconscious by a continuous flow of sea water (0 to 5° C) containing 0·05% 2-phenoxyethanol over their gills. Heparin sodium, 1 mg/ml saline, was used as an anticoagulant during surgery. Groupers were cannulated using the technique of Soivio *et al.* [31] with the modifications of Iwama and Ishimatsu [22]. Each fish was quickly transferred to an individual tank and maintained free-swimming without any further handling. Fish were fed twice daily. One ml of blood samples were collected from each experimental fish 1, 3, 5, 7, 9, 11 and 13 days after cannulation.

Non-cannulated anaesthetised fish were sham-punctured using a 20 gauge needle but without withdrawing any blood on day 0 (the same time the other fish were cannulated). Each fish was kept separately in individual tanks. The same volume of blood samples was taken on the same days as cannulated fish but by caudal vessel puncture. These fish were fed as for the cannulated fish and thus not starved prior to anaesthetisation and venous puncture, which took 10–15 min from netting and anaesthetising the fish to obtaining the blood sample.

HEMATOLOGICAL ANALYSIS

Blood parameters

To count the total number of erythrocytes and leukocytes, 20 µl blood was placed in a Microcell counter (Sysmes F-800, Japan) following the manufacturer's recommended protocol.

Cortisol determination

Approximately 500 μl blood from each sample were centrifuged at 735 × *g* for 10 min at 4° C. The plasma was separated and stored at − 20° C. Cortisol level was measured by a radio-immunoassay, as described in Chang and Yueh [32]. Synthetic human cortisol 1, 2, 4, 8, 16 ng/500 μl served as standards and were processed in the same manner as the unknown plasma.

Leukocyte isolation and phagocytic index

Peripheral blood leukocytes (PBL) were isolated as described by Miller *et al.* [33] using lymphoprep (density 1·077 g/ml) (Gibco-BRL). Based on trypan blue exclusion criterion, the cells used in the experiments were more than 92% viable. PBL were resuspended and their concentration was adjusted to 3×10^6/ml in Aim V-Leibovitz L15 medium (Gibco) supplemented with 5·5 mM glucose (ALG). Using a high salt solution, the osmolarity of AL and ALG media were adjusted to 375 ± 10 mOsm/kg prior to use.

The PBL phagocytic index was determined using a 96-well microtitre plate assay, modified as described by Law *et al.* [34]. FITC-labelled latex beads (0·105 μm in diameter) (Sigma) and PBL (10–12 μm in diameter) were mixed 50 000:1, a ratio found to be optimal in a preliminary experiment. The mixture was incubated in the dark, at 25° C for 120 min. Trypan blue solution (125 μg/ml) was added to the mixture and allowed to react for 10 min to quench the fluorescent signal of the extra-cellular beads. Fluorescent signal units (FSU) were measured using a fluorescence intensity analyser (Fluorite 1000, Dynex) using a 485/530 nm channel. Control wells were processed similarly, but ALG medium replaced the PBL. Three wells were measured for each sample. The PBL phagocytic index (%) was defined as

$$(\mathrm{Fp} - \mathrm{Fo}/\mathrm{Fb} - \mathrm{Fo}) \times 100$$

where Fo=control well FSU, Fb=the experimental well FSU of PBL isolated from grouper prior to surgery, and Fp=the experimental well FSU of PBL isolated from grouper following surgery.

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA), followed by Duncan's multiple range test at 5% confidence levels, was conducted to test the significant difference between non-OTC and OTC treatments. ANOVA, followed by Student's *t*-test, was used to analyse the differences between the pre- and post-cannulated fish; and between the pre- and post-venous puncture fish. The

same ANOVA with Student's *t*-test was used again to analyse the difference between venous puncture and cannulation after OTC treatments. All data are expressed as a mean ± standard error of the mean (S.E.M.).

III. Results

There were five treatments in this experiment: (1) unstressed fish from which the baseline blood samples were collected from caudal vessel puncture, (2) caudal vessel puncture non-OTC-treated, (3) caudal vessel puncture OTC-treated, (4) cannulated non-OTC-treated and (5) cannulated OTC-treated.

Plasma cortisol levels increased significantly in non-OTC- and OTC-treated grouper within 24 h after a sham-puncture on day 0 (Fig. 1A). Cortisol levels continued to increase in non-OTC-treated grouper to a maximum of 891 ng/ml, approximately 26-fold that of an unstressed fish. Plasma cortisol levels in OTC-treated, venous puncture grouper peaked at 505 ng/ml on day 7, a 14-fold increase (Fig. 1A). Within 24 h of surgery, cortisol levels in non-OTC-treated, cannulated grouper increased to 88 ng/ml. This was significantly greater than the pre-cannulation level of 30 ng/ml. Cortisol levels continued to increase dramatically in non-OTC-treated fish to a maximum of 951 ng/ml 3 to 5 days after cannulation, a 32-fold increase. In contrast, plasma cortisol level in OTC-treated, cannulated grouper did not change within 24 h of surgery. It peaked at 178 ng/ml on days 3 to 5, only a 6-fold increase. Plasma cortisol level returned to normal on day 7 in OTC-treated grouper and on day 9 for non-OTC-treated grouper in the cannulated group (Fig. 1B).

The total RBC counts in non-OTC- and OTC-treated, venous punctured grouper were never significantly different from each other. However, the total RBC count for both groups decreased significantly from day 7 to day 13 (Fig. 2A). The total RBC count in non-OTC- and OTC-treated, cannulated grouper decreased significantly from days 1 to 5 after cannulation, but quickly returned to normal level in OTC-treated cannulated grouper on day 7 (Fig. 2B). The total WBC count in non-OTC- and OTC-treated, venous punctured grouper increased significantly from day 1 to day 13. In the non-OTC treated grouper, the total WBC count increased markedly from $4{\cdot}74 \times 10^7$ (day -7) to $7{\cdot}08 \times 10^7$ (day 11) cells/ml (Fig. 3A). The total WBC count in non-OTC-treated, cannulated grouper were significantly greater after treatment than before (day -7). However, cannulated groupers treated with OTC exhibited significantly elevated WBC counts only on days 1, 3 and 5 (Fig. 3B). From days 7 to 13, the total RBC and WBC counts in OTC-treated, cannulated grouper were significantly different from the counts in OTC-treated, venous punctured grouper.

The phagocytic index of peripheral blood leukocytes (PBL) in unstressed grouper was set as the 100% baseline. The phagocytic index of non-OTC-treated, venous punctured grouper decreased to a low of 50% on day 5 and remained low until day 13. In OTC-treated fish, the phagocytic index returned to normal on day 11 (Fig. 4A). No major changes were observed in the phagocytic index on day 1 after dorsal aorta cannulation. However, the PBL phagocytic index in non-OTC-treated fish had decreased significantly by day 3 and continued to decrease to a low of 46% on day 7. The phagocytic index

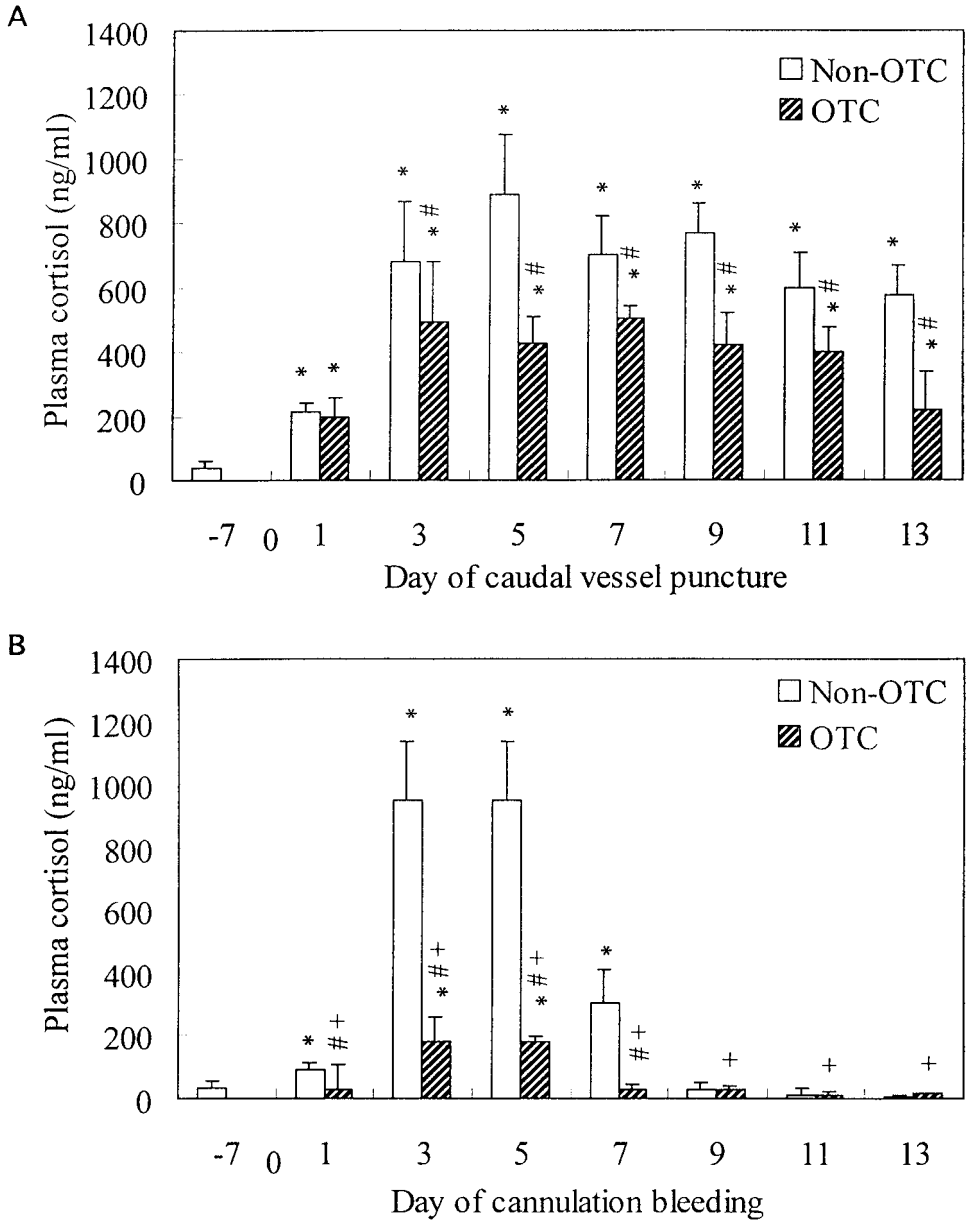

Fig. 1. A. Effect of blood sampling by caudal vessel puncture and immersion with 20 ppm oxytetracycline (OTC) on plasma cortisol level. B. Effect of cannulation and post-surgery treatment with 20 ppm OTC on plasma cortisol level. The vertical line shows the SEM for each bar (n=6). An asterisk indicates a significant difference (P<0·05) from the blank (pre-treatment), the # indicates a significant difference (P<0·05) from the respective control (without OTC treatment) and the + indicates a significant difference (P<0.05) from the venous punctured fish in the presence of OTC.

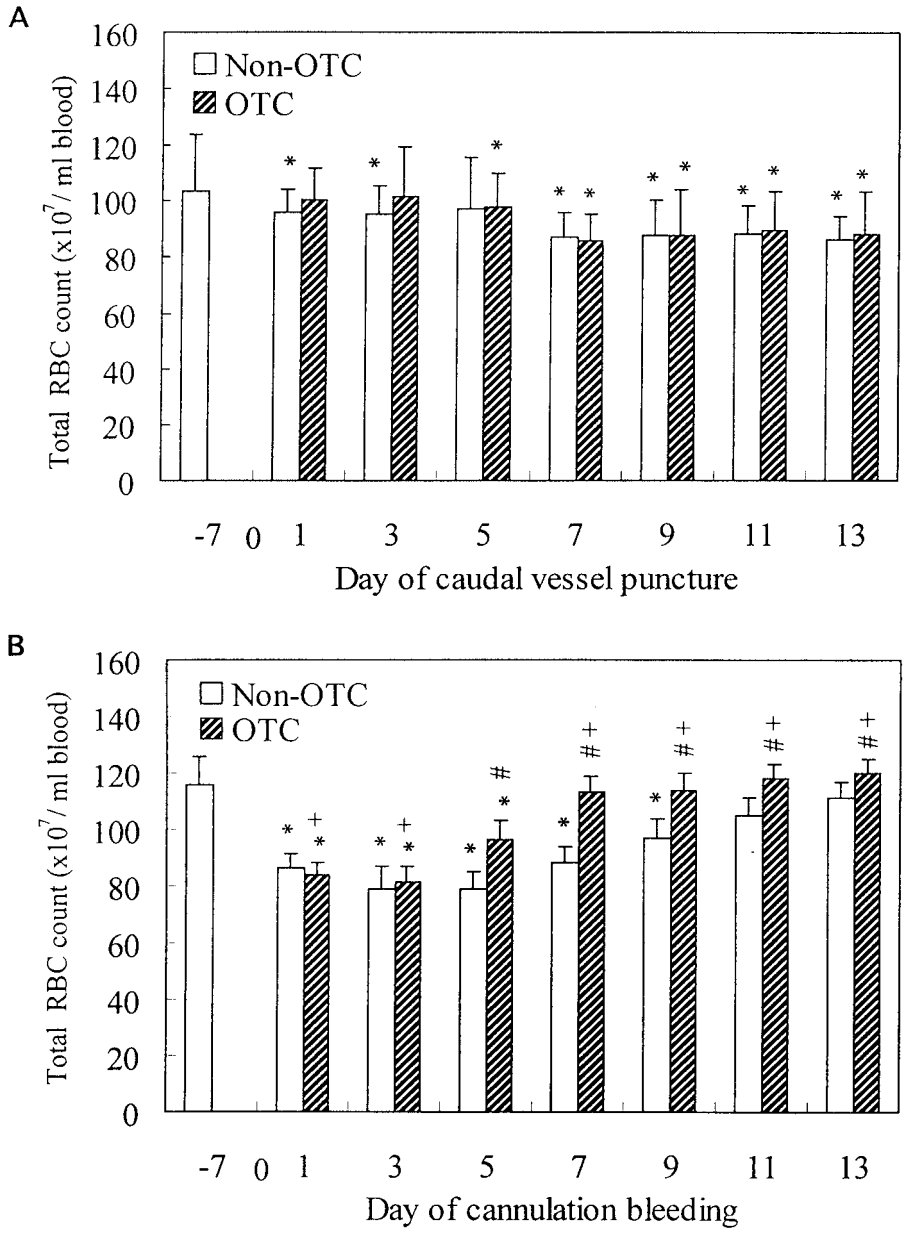

Fig. 2. A. Effect of blood sampling by caudal vessel puncture and immersion with 20 ppm oxytetracycline (OTC) on total RBC count. B. Effect of cannulation and post-surgery treatment with 20 ppm OTC on total RBC count. The vertical line shows the SEM for each bar (n=6). An asterisk indicates a significant difference (P<0·05) from the blank (pre-treatment), the # indicates a significant difference (P<0·05) from the respective control (without OTC treatment) and the + indicates a significant difference (P<0·05) from the venous punctured fish in the presence of OTC.

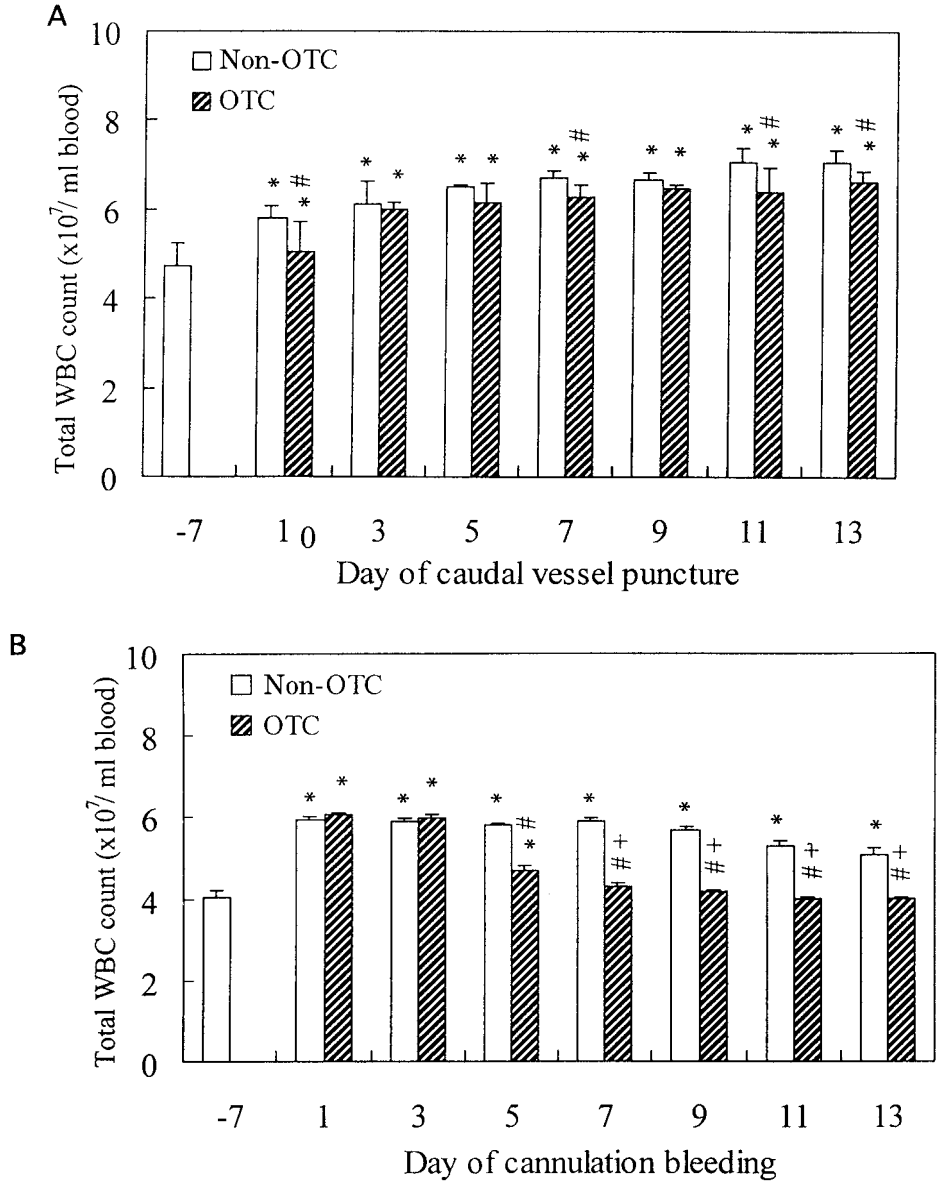

Fig. 3. A. Effect of blood sampling by caudal vessel puncture and immersion with 20 ppm oxytetracycline (OTC) on total WBC count. B. Effect of cannulation and post-surgery bath with 20 ppm OTC on total WBC counts. The vertical line shows the SEM for each bar (n=6). An asterisk indicates a significant difference (P<0·05) from the blank (pre-treatment), the # indicates a significant difference (P<0·05) from the respective control (without OTC treatment) and the + indicates a significant difference (P<0·05) from the venous punctured fish in the presence of OTC.

gradually increased after day 7, but failed to return to normal by the end of the experiment (day 13). In OTC-treated, cannulated grouper, the phagocytic index began to recover on day 5 and returned to normal by day 11 (Fig. 4B).

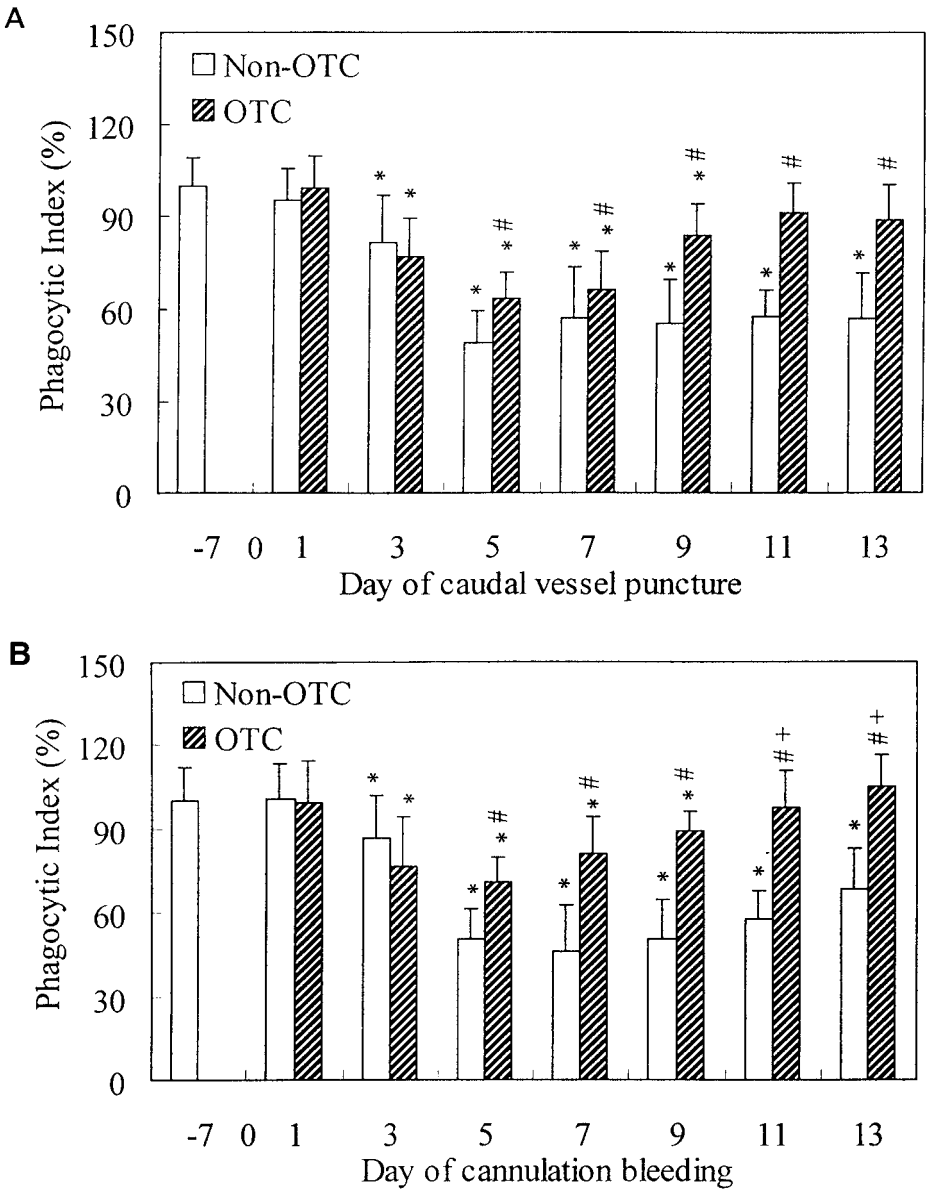

Fig. 4. A. Effect of blood sampling by caudal vessel puncture and immersion with 20 ppm oxytetracycline (OTC) on phagocytic index. B. Effect of cannulation and post-surgery treatment with 20 ppm OTC on phagocytic index. The vertical line shows the SEM for each bar (n=6). An asterisk indicates a significant difference (P<0·05) from the blank (pre-treatment), the # indicates a significant difference (P<0·05) from the respective control (without OTC treatment) and the + indicates a significant difference (P<0·05) from the venous punctured fish in the presence of OTC.

The phagocytic index of OTC-treated, cannulated grouper was significantly greater than that of OTC-treated, venous punctured grouper on days 11 and 13. Immersing groupers in OTC solution, which decreased the recovery period, mitigated the adverse effects of surgery.

IV. Discussion

The baseline values for total RBC and WBC counts, and plasma cortisol levels in the groupers used in this study fell within the ranges reported for other species of unstressed fish, including brown trout [35], rainbow trout [22], tilapia [36], carp [16], gilthead sea bream [37] and sea bass [38].

Taking blood samples no greater than 0·5 to 0·7% of body weight at 1-week intervals does not harm fish [39]. In addition, blood samples up to 2% of body weight can be collected at 4-week intervals without compromising the immunological performance of fish lymphocytes [33]. In this study, we withdrew 1·0 ml blood (0·17% of body weight) from groupers with an average weight of 605 g, 7 days prior to cannulation surgery or venous puncture. Thus, we do not believe blood withdrawal on day -7 will cause significant, stress-induced haematological changes in the groupers used in this study prior to surgery or venous puncture.

Due to the extreme sensitivity of the hypothalamo-pituitary-interrenal axis, many researchers use blood corticosteroid level as one indicator of stress. A variety of adverse stimuli, including pollutants, heat, exercise and hypoxia, pharmacological agents, including anaesthetics, and physical stressors, such as the handling, restraint, and transportation of fish, can increase blood cortisol levels. Although we did not determine the level of plasma cortisol on day 0, it was a remote possibility that the puncture procedure itself was more stressful than the surgery procedure. This was suggested by the finding that the changes in plasma cortisol that occurred in the OTC-treated punctured fish by 24 h after the sham-puncture are significantly bigger than the changes in the OTC-treated surgery group. This may be explained by the fact that the punctured fish underwent twice anaesthetisation and associated handlings by day 1, in comparison to single anaesthetisation and associated handling in the surgery group. The results demonstrate that repeated anaesthetisation, its associated handling and possible internal bleeding caused by the venous puncture stressed the fish more than the cannulation and the withdrawal of blood samples via the cannula. Plasma cortisol in non-OTC-treated, cannulated groupers increased approximately 3-fold on day 1. A similar increase followed stress in tilapia [40]. We believe the 3-fold increase in groupers also represents mild stress. However, the 31-fold increase in cortisol level in non-OTC-treated, cannulated grouper on days 3 through 5 cannot be categorised as mild only. Plasma cortisol level returned to normal on day 9. Dogfish recovered more quickly, but still showed signs of stress 24 h post-operation [17]. In tilapia, plasma cortisol returned to normal levels in 2 to 3 days [28]. Freshwater trout recovered more quickly than other species: plasma cortisol returned to near control levels within 24 h of successful dorsal aorta cannulation [22]. In grouper treated with OTC immediately following cannulation and blood withdrawing from the cannula, cortisol levels

decreased to near normal levels much faster than in non-OTC-treated fish. Thus, to minimise fish stress, it is crucial that fish be held in a carefully controlled environment before, during and after blood sampling and operations.

Cannulation surgery and blood sampling from the caudal vessels caused significant decreases in the RBC count and significant increases in the WBC count. RBC decreased significantly immediately after cannulation. However, no grouper lost significant amounts of blood during cannulation and no fish died after surgery, indicating there was little, if any, internal haemorrhaging. Nevertheless, surgery mechanically damaged some tissue and cannulation can cause adhesive thrombosis [13]. The significant increase in total WBC count 24 h following surgery suggests that grouper might have experienced inflammation induced by mechanical damage and bacterial infection. However, histopathological examination did not provide evidence to support either possibility. However, OTC treatment reduced the increase in WBC, providing indirect evidence that infection contributed to the WBC increase. Afonso *et al.* [41] observed the rapid influx of neutrophils that peaked at 24 to 48 h post-injection of live bacteria in the peritoneal cavity of rainbow trout. RBC and WBC counts for the cannulated grouper returned to normal sooner in OTC-treated than in non-OTC treated fish. In both OTC-treated groups the RBC and WBC counts for venous punctured grouper were significantly different from those for cannulated groupers. The blood parameters of cannulated groupers changed more rapidly than those of venous punctured grouper. They always differed significantly from normal on day 1 and gradually returned to normal in 2 weeks. In contrast, the results demonstrate that repeated anaesthetisation, the associated handling and the possible internal bleeding of the venous punctured groupers stressed the fish more than cannulation and the withdrawal of blood samples via the cannula.

OTC has immunosuppressive effects in fish [42]. The primary, not the secondary immune response was depressed 80–95% in OTC-treated carp [43]. OTC might inhibit macrophage processing of antigens, decreasing stimulation of the immune system [44]. OTC (10 ppm, intraperitoneal injection) reduced the activity of the nonspecific and specific immune system compartments of salmonids [45]. To minimise the prompt, direct effects of OTC on haematological parameters, after surgery we immersed each grouper in sea water containing 20 ppm OTC. OTC suppresses the phagocytic activity of fish macrophages *in vitro* [46]. However, PBL phagocytic activity in OTC-immersed grouper was greater than in non-OTC-treated fish, although the PBL phagocytic index for both groups was significantly lower than before surgery index. The antimicrobial properties of OTC apparently compensated for its immunosuppressive effects.

The PBL phagocytic index did not change notably on day 1 post-cannulation, but it did decrease by 3 days post-cannulation, despite an increase in the total WBC count on days 1–3. In our study PBL phagocytic activity enhancement in OTC-treated fish indicates a qualitative improvement. Because OTC has no known side effects in grouper, it can be used to prevent infection after invasive surgery. Sources of infection, such as poor water quality, non-aseptic surgical conditions, feed pellets, faeces, and other

wastes, can be difficult to eliminate in an open tank system. Adding OTC to post-surgery tank water is a convenient and practical method for preventing bacterial infection. Although hygienic conditions and aseptic procedures are a primary consideration, OTC treatment is highly recommended before, during and after invasive surgery.

Cannulation was successful because no groupers died from surgery and we were able to extract blood through the cannulae of all cannulated groupers every 2 days until the end of the experiment. Experimental fish were sacrificed 14 days after surgery, thus we do not know how long cannulated grouper can survive and be available for blood sampling. Tilapia with cannulated dorsal aorta survived at least 40 days [28]. European eels with a cannulated ventral aorta and Altantic salmon with a cannulated dorsal aorta survived 3 to 4 months [13, 18] whereas cannulated carp survived up to 200 days (Ishimatsu, personal communication).

The low cortisol level on day 1 post-cannulation indicated that dorsal aorta cannulation was only mildly stressful to grouper. Subsequent tissue inflammation induced by mechanical damage caused by surgery was also mildly stressful. In contrast, microbial infection may have been extremely stressful as OTC treatment significantly reduced plasma cortisol level by days 3 and 5 post-cannulation. Bacterial infection resulting from non-aseptic conditions, rather than the direct effect of the dorsal aorta cannulation procedure, may thus have caused most of the observed changes in haematological parameters. Blood samples from cannulated grouper were obtained quickly and easily because we did not have to net the fish first. Taking blood samples did not visibly disturb the fish. Therefore, dorsal aorta cannulation permits blood samples to be taken repeatedly, without stressing the fish unduly, and should prove very useful for immune studies in fish.

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