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(Notes)

The Effects of Acute Osmotic Stress on Innate Immunity of Nile Tilapia (*Oreochromis niloticus*)

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The effects of osmotic stress on the non-specific immune response of Nile tilapia, *Oreochromis niloticus*, were investigated. Osmoregulatory mechanism of tilapia has been studied, but less information is available about innate immune response of *O. niloticus* faced with hyperosmolality. Acute osmotic stress was elicited by transferring tilapia from freshwater (FW) to 24 psu seawater (SW). Non-specific immune parameters including lysozyme activities of plasma and head kidney (HK), alternative complement pathway (ACP) activity in plasma, phagocytic capacities of spleen and HK immune cells, and respiratory burst activity of immune cells in both HK and spleen were analyzed. Lysozyme activities were increased at 1 h and 30 h after transfer to SW, but decreased at 10 h after SW transfer. Conversely, ACP activity increased 10 h after SW transfer. Phagocytic capacity increased slightly at 1 h and 5 h after SW transfer, and respiratory burst activity showed an increase in superoxide release at 10 h after SW transfer. Taken together, these results indicate that the exposure of tilapia to hyperosmotic conditions has immunostimulatory effects on cellular and humoral immune reactions.

Key words: Respiratory burst activity, ACP, Phagocytosis, Lysozyme activity, *Oreochromis niloticus*

Introduction

Environmental stimulations such as salinity changes and sudden temperature changes exert negative effects on fish physiology, psychology, growth, and breeding (Crawshaw, 1979; Cuesta et al., 2005). Weng et al. (2002) have reported that tilapia can avoid dehydration and adapt to the hypero-smolality of seawater (SW), making tilapia ideal for investigating osmoregulation in teleosts. In the past, most studies of tilapia have focused on their osmoregulation, but knowledge of the immunological responses of fish facing hyperosmolality is limited.

Vertebrates have well-developed immune systems comprising both acquired and innate immunity; innate immunity protects hosts against invading foreign pathogens in a non-specific manner. Like tilapia (*O. niloticus*), other species of FW fish (catfish, *Ictalurus punctatus*; carp, *Cyprinus carpio*; rainbow trout, *Oncorhynchus mykiss*) have natural killer cells referred to as non-specific cytotoxic cells (NCC) (Jaso-Friedmann et al., 2000). NCCs are important in providing resistance against bacterial pathogens such as *Streptococcus iniae* in tilapia (Taylor et al., 2001). Lysozyme, a bactericidal peptide, is an important component of the immune defenses of both FW and marine fish species (Lie et al., 1989). Increases in lysozyme activity, phagocytosis, and cell meditated cytotoxicity, and a decrease in antibody production were found in salmon during FW to SW transfer (Marc et al., 1995). Chronic exposure of tilapia to 0.53% tannery effluent (TE) at low temperature significantly reduced antibody responses, non-specific lysozyme activity, and the production of reactive nitrogen and oxygen species (Prabakaran et al., 2007). It had been reported previously that humoral immune responses in gilthead sea bream were increased under conditions of hyperosmolality (Cuesta, et al., 2005). In tilapia, the effects of acute salinity stress on immune responses are unclear. In this study, *in vivo* tests were conducted to evaluate the immune responses of tilapia during environmental changes. Hence, this study aims to determine the effects of salinity stress on the phagocytic activity,

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respiratory burst (release of superoxide anions), lysozyme activity, and activity of the alternative complement pathway of tilapia, *O. niloticus*.

Materials and Methods

Fish

Nile tilapia *O. niloticus* of approximately 30-40g (about 6 months old) were obtained from the fish farm at Kunsan National University and maintained in FW recirculation tanks supplied with a filter and aeration system at 22-26°C. Approximately 60 fish were used for this experiment. For hyperosmolality (SW) challenge tests, four treatment times (1, 5, 10, and 30 h) were used, with five fish per exposure time maintained in different tanks. Two additional tanks of fish were kept in FW as controls, and five fish were removed and sampled at the same times as experimental groups. At the end of the experiment, tilapia were immediately anesthetized and sacrificed on ice; blood, HK, and spleen were collected.

In all tests, the fishes were fed daily on a commercial diet (Purina, Kunsan, Korea) during the experiment. Prior to the experiments, fish were acclimated to laboratory conditions for more than 1 month, and both male and female tilapia ranging from 35 to 65 g (mean ± SD = 50 ± 20 g) with no significant differences among the SW treatments were used in these experiments. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals of Kunsan National University. Twenty-two psu SW was prepared using tap water with artificial sea salt (Coral Marine Sea Salt®, Aqua Craft, Inc. Products®, Hayward, CA, USA). Salinity was measured using a salinity refractometer (Hand Refractometer S/Mill-E, Atago, Itabashi-Ku, Tokyo, Japan).

Blood sampling

Tests were conducted on 5 fish at each of four exposure times (1, 5, 10, and 30 h), with fish sampled at each time point being maintained in individual tanks. Five fish per group were carefully netted and anesthetized with 0.02% benzocaine solution, and blood was collected from the caudal vessel using a syringe pretreated with heparin ammonium (400 U/mL) (Sigma, St. Louis, MO, USA). The majority of the collected blood was transferred to 1.5 mL centrifuge tubes and stored on ice. Plasma was obtained by centrifugation of blood samples at 9,600×*g* for 5 min and subsequently used for lysozyme activity and ACP analyses.

Preparation of leukocytes

Spleen and HK from both FW and SW transfer fish were used to prepare leukocytes. Cell suspensions were prepared in an RPMI-1640 (Gibco) medium by grinding cells with ground glass and filtering them through a nylon mesh. Leukocytes were separated on a 51% Percoll gradient (Sigma) in PBS (Gibco). Cells collected at the interface were washed twice and resuspended with RPMI-1640 medium, and the cell suspensions were then centrifuged at 500×*g* at RT for 10 min. Finally, the cells were washed twice with PBS.

Analysis of lysozyme activity

HK and plasma lysozyme activity was measured in triplicate for each of five fish per sampling time. Samples (200 μL) of head kidney suspension with RPMI-1640 medium in a 1.5 ml centrifuge tube were homogenized and centrifuged at 9600×*g* for 5 min. The supernatant was kept for lysozyme assay. HK and plasma lysozyme activity were determined using a turbidimetric assay (Shugar, 1952). *Micrococcus lysodeikticus* (0.4 mg/mL; Sigma) was suspended in 0.05 M potassium phosphate buffer (pH 6.2) and used as a substrate. HK and plasma (10 μL) were added to 260 μL of bacterial suspension and incubated for 5 min at 28 ± 1°C; the reduction in absorbance was measured at 490 nm using an automated spectrophotometer (SpectraMax 190). One unit of lysozyme activity was defined as the amount of plasma and HK required to cause a reduction in absorbance of 0.001/min. The data are presented herein as ratios of the FW fish sampled at each experimental time ± SD (n=5).

Phagocytic activity

To measure the phagocytic activity of leukocytes, fish were transferred to 24 psu SW and to FW. At 0, 1, 5, 10, and 30 h after transfer, five tilapia per group were anesthetized, and the HK and spleen were removed; leukocytes were prepared as outlined above. Flow cytometry (Esteban et al., 1998) was used to measure the phagocytic activity of HK cells and splenocytes. To each 100 μL sample of HK or spleen leukocyte suspension, aliquots of 10 μL FITC-labeled *Escherichia coli* JM 109 (adjusted to 1×10^8 cells/mL) were added in 1.5 ml tubes. The samples were then centrifuged (300×*g*, 5 min, 22°C), resuspended in RPMI-1640 medium, and incubated at 22°C for 45 min. At the end of the incubation, the samples were placed on ice to stop phagocytosis, and 800 μL of ice-cold PBS were added to each sample, and analysis by flow cytometry (Cytomics™ FC500,

Beckman, Fullerton, CA, USA) was conducted. Instrument settings were adjusted to obtain optimal discrimination of the different cell populations present in the HK and spleen leukocyte suspensions. Only the phagocyte population was acquired by cell size and granularity and analyzed for each sample. Analyses were performed on 10,000 cells that were acquired at a rate of 300 cells/s. Data were collected in the form of two-parameter side scatter (granularity) (SSC) and forward scatter (size) (FSC) or green fluorescence (FL1) dot plots. Fluorescence histograms were obtained from a computerized system showing relative fluorescence on a logarithmic scale. Phagocytic capacity was defined as the relative number of ingested bacteria per phagocytic cell assessed from the mean fluorescence intensity of the cells. Data are presented herein as ratios of the FW fish sampled at each experimental time as mean ± SD (n=5).

Respiratory burst assay

The respiratory burst activity of HK and spleen leukocytes was studied by flow cytometry as previously described (Craig et al., 2005). Samples (100 μL) of HK and spleen leukocyte suspensions were mixed with 0.05 M dichlorofluorescein diacetate (DCFH-DA, Sigma) in the dark on a shaker platform (55 rpm) at room temperature for 25 min. In the presence of reactive oxygen species, DCFH-DA is oxidized to dichlorofluorescein (DCF), which can be detected by flow cytometry, thus providing a quantitative assessment of the respiratory burst in individual cells. No stimulatory agents were added to analyze respiratory burst activity. After incubation, the cells were washed twice with cold PBS and resuspended in 500 μL of cold PBS for analysis by flow cytometry, which was performed within 30 min. Cells with a fluorescence intensity greater than background were considered positive, indicating that a respiratory burst occurred. The respiratory burst activity per cell was assessed from the mean fluorescence intensity of the cells. Data are presented herein as ratios of the FW fish sampled at each experimental time as mean ± SD (n=5).

Alternative complement activity

The activity of the alternative complement pathway (ACP) was assayed using rabbit red blood cells (RRBC) as targets. RRBC were washed in phenol red-free HBSS (Gibco) without Mg^{2+} and Ca^{2+} and 10 mM EGTA (Sigma) and resuspended at 3% (v/v) in HBSS with the addition of 0.5 mM Mg^{2+}. Plasma samples were diluted (1:50) to different volumes ranging from 0.1 mL to 0.25 mL and were dispensed into a series of test tubes. The total volume was brought up to 0.25 mL with the same buffer and was then added to 0.1 mL of RRBC suspension. After incubation for 1 h at 22°C, samples were centrifuged at 300×g at 4°C for 5 min to remove unlysed erythrocytes. The relative hemoglobin content of the supernatants was assessed by measuring their optical density at 540 nm using an automated microtiter plate spectrophotometer. Values for maximum (100%) and minimum hemolysis were obtained by adding 50 μL of distilled water or HBSS to 25 μL samples of RRBC. The degree of hemolysis (Y) (percentage of hemolytic activity with respect to the maximum) was calculated, and a lysis curve for each sample was obtained by plotting $Y/(1-Y)$ against the volume (mL) of plasma added on a $\log_{10}$-$\log_{10}$-scaled graph. For each experimental group, the volume of plasma producing 50% hemolysis (ACH_{50}) was determined, and the number of ACH_{50} (50% hemolysis/ml serum) units/mL was calculated. The data are presented herein as ratios of the FW fish as mean ± SD (n=5).

Statistical analyses

Experimental data are presented as mean ± SD. Comparisons between control and treatments were examined by one-way ANOVA followed by least square difference (LSD) t-tests (SAS 2004). Differences among treatment groups at different times were tested using the least significant difference test. An asterisk (*) denotes a P-value <0.05, indicating a significant difference compared with the control group.

Results

Lysozyme activity

As shown in Fig. 1A, production of lysozyme activity in plasma was increased (P<0.05) at 1 h and 30 h after SW transfer, but was decreased 10 h after SW transfer. One sample of HK tissue was insufficient for lysozyme analysis; hence, three HK tissue samples were pooled in this assay. Profile of HK lysozyme activity (Figs. 1A and B) correlated with lysozyme activity of plasma.

Alternative complement activity

Complement activity in plasma was analyzed after the transference of tilapia to 24 psu SW. After 1 h, complement activity was decreased compared with FW controls. Surprisingly, after 10 h, complement activity was increased compared with FW controls, even though this trend was not sustained through 30 h (Fig. 2).

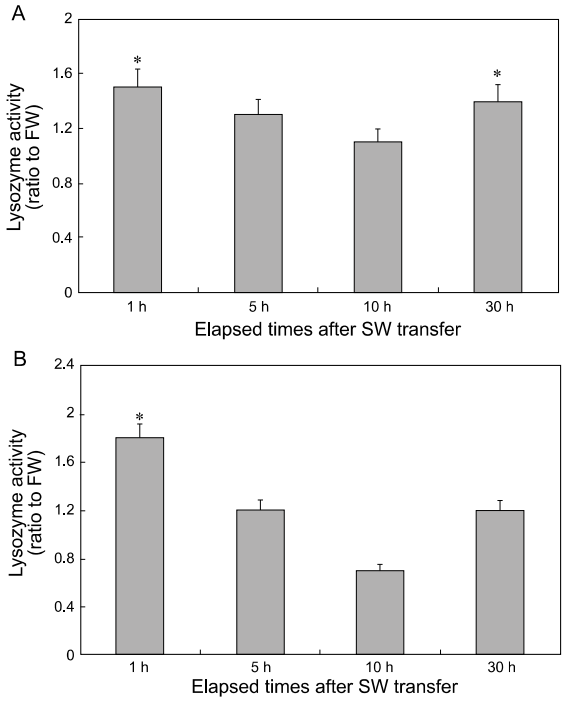

Fig. 1. *In vivo* lysozyme activity in (A) plasma and (B) HK after tilapia were exposed to 24 psu SW for 1, 5, 10 and 30 h. Data are expressed as mean±SD (n=5). Asterisk denotes P value (P<0.05), which means the data are significantly different from the control group.

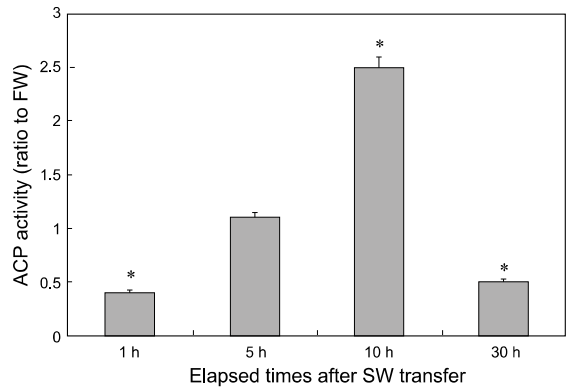

Fig. 2. *In vivo* plasma ACP activity after tilapia were exposed to 24 psu SW for 1, 5, 10, and 30 h. Data are expressed as mean±SD (n=5). Asterisk denotes P value (P<0.05), which means the data are significantly different from the control group.

Immune responses by HK cells after SW transfer

Phagocytic capacity, measured as the intensity of fluorescence, was increased 5 h after SW transfer in HK (Fig. 3A). Respiratory burst activity was significantly increased at 10 h (P<0.005) and significantly decreased at 30 h after SW transfer (Fig. 3B).

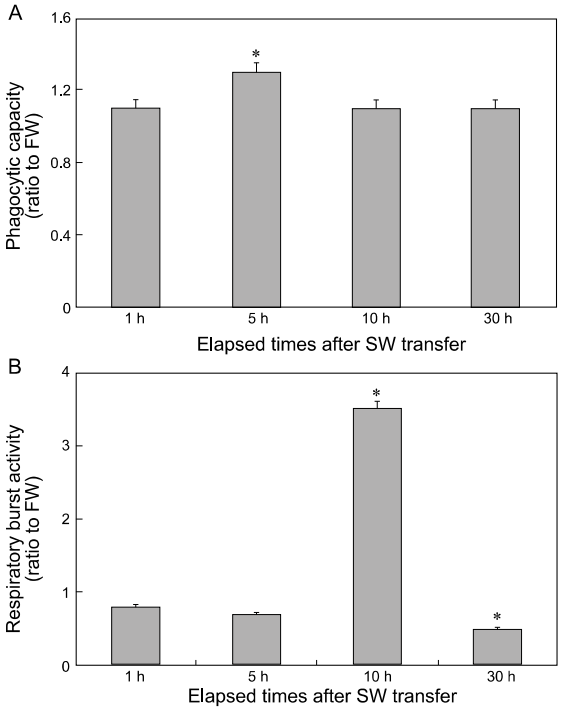

Fig. 3. *In vivo* cellular parameters of HK: (A) phagocytosis capacity measured as intensity of fluorescence; (B) respiratory burst activity (intensity of respiratory burst) after tilapia exposure to 24 psu SW for 1, 5, 10 and 30 h. Asterisk denotes P value (P<0.05), which means the data are significantly different from the control group.

Immune response by splenocytes following SW transfer

Phagocytic capacity was increased 1 h (P<0.05) after SW transfer (Fig. 4A). Intensity of respiratory burst activity was elevated at 10 h (P<0.05), but decreased at 30 h after SW transfer (Fig. 4B).

Discussion

Many species of fish suffer stress responses to sudden transfer from FW to SW, and stress is often associated with the suppression of immune parameters. In non-smolting salmonids, FW-SW and FW-FW transfer resulted in a typical acute stress response characterized by a marked increase in plasma cortisol concentration (Taylor et al., 2007). The concentrations of cortisol and plasma osmolality are increased markedly after transfer from FW to SW and exhibit a significant positive correlation (Kajimura et al., 2004). However, tilapia are reported to be capable of direct transfer from FW to 25 psu SW with certain physiological effects (Weng et al., 2002), but there is

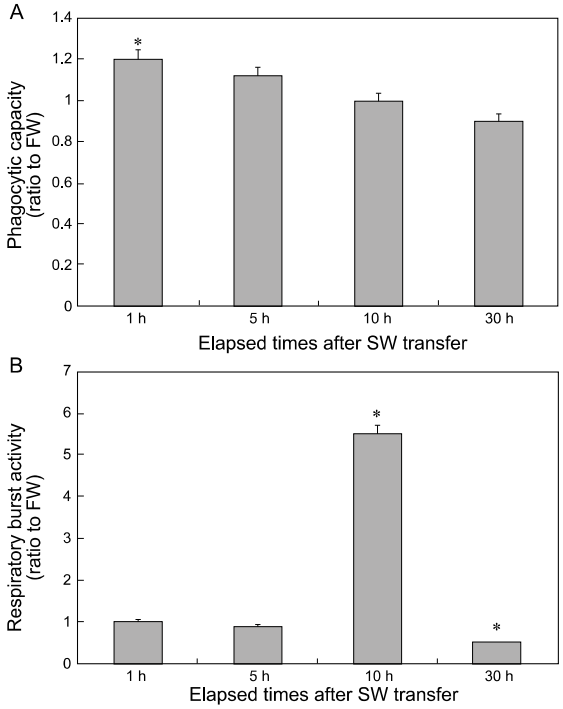

Fig. 4. *In vivo* cellular parameters of spleen: (A) phagocytosis capacity measured as intensity of fluorescence; (B) respiratory burst activity (intensity of respiratory burst) after tilapia exposure to 24 psu SW for 1, 5, 10 and 30 h. Data are expressed as mean ± SD (n=5). Asterisk denotes P value (P<0.05), which means the data are significantly different from the control group.

no available information on the effects of such transfer on innate immune parameters. The present study was thus conducted to investigate the effects of salinity stress on the innate immune responses of tilapia.

In general, humoral innate factors like lysozyme and complement are important antimicrobial factors. The greatest concentration of lysozyme activity is found in the HK of fish due to the HK's high numbers of leukocytes (Marc et al., 1995; Lee et al., 1998; Takahashi et al., 1986; Yousif, et al., 1991; Roed et al., 1993). Nile tilapia cultured in 24 psu salinity for 2 and 4 weeks, or in 12 psu salinity for 4 weeks, showed increased lysozyme activity (Dominguez, et al., 2005). In our study, plasma and HK lysozyme activity were increased at 1, 5, and 30 h after tilapia were transferred to SW. Surprisingly, the lowest lysozyme activity was observed 10 h after SW transfer, and this was correlated with an increase in plasma ACP activity. ACP, a non-specific humoral parameter, exhibits lytic, proinflammatory, chemotactic, and opsonic activities in teleosts (Ellis, 1999). Following acute stress in teleosts, both enhancement and suppression of plasma lysozyme activity have been reported depending on the type, intensity, and duration of the stressor (Fevolden et al., 2003). However, the exact mechanisms by which both lysozyme and ACP activities can show opposite responses depending on stress conditions remains to be studied further.

The phagocytic activity of head kidney leukocytes increased in brown trout (*Salmo trutta*) transferred from FW to SW (Marc et al., 1995), whereas in the present study, we found that tilapia HK leukocytes and spleen cells exhibited no detectable changes in phagocytosis compared with control fish after 24 psu SW transfer. Alteration in the numbers and composition of circulating leukocytes is an indicator of acute and chronic stress (Barton and Iwama, 1991). When phagocytic capacity was compared between HK leucocytes and splenocytes, an increase in splenocyte phagocytic capacity was observed after 1 h of SW transfer, but increases in HK phagocytic capacity were found only after 5 h of SW transfer. These facts suggest that a hyperosmotic medium can induce phagocytic activity in tilapia at earlier elapsed times after SW transfer.

Non-smolting rainbow trout HK immune cells showed greater respiratory burst activity (approximately 50% higher) when transferred to SW (Taylor et al., 2007). It has been reported previously that stress affects the phagocytic and/or respiratory burst activity of spleen, head kidney and blood leucocytes (Thompson et al., 1993; Pulsford et al., 1994; Vazzana., 2002; Liebert and Shreck, 2006). In our experiments, the respiratory burst activities of tilapia HK leucocytes and spleen cells were increased after 10 h of SW transfer. These results were similar to those for ACP. However, further studies should be performed to investigate whether respiratory burst activity is correlated with ACP in tilapia after SW transfer.

The values of each immune parameter measured at different elapsed times after SW transfer did not show constant increases. However, the transfer of *O. niloticus* from FW to SW caused partial increases at specific SW transfer times in various immune parameters, suggesting that the exposure of tilapia to hyperosmotic conditions has an immunostimulatory effect on these innate cellular and humoral immune parameters.

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