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Respiratory Burst Activity in Head Kidney and Spleen Leukocytes of Tilapia (*Oreochromis mossambicus*) under Acute Osmotic Stress

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Bharath Kumar-Velmurugan, Ing-Fong Jiang, Hung-Yuan Shih, Der-Nan Lee, and Ching-Feng Weng (2012) Respiratory burst activity in head kidney and spleen leukocytes of tilapia (*Oreochromis mossambicus*) under acute osmotic stress. *Zoological Studies* **51**(8): 1290-1297. Tilapia *Oreochromis mossambicus* is capable of surviving in a wide range of salinities and temperatures. In the present study, we determined how salinity influences the respiratory burst (RB) activity of tilapia. At first, tilapia were exposed to freshwater (FW) or 25 ppt seawater (SW), and serum was isolated from these groups. Further, leukocytes of the head kidneys (HKs) and spleen were isolated from tilapia exposed to 300 or 500 mOsm osmolality. Following osmolality exposure, cells were incubated (1) without serum (as the control), (2) with serum of tilapia exposed to fresh water (FW), and (3) with serum of tilapia exposed 25 ppt seawater (SW). A cell viability assay indicated that exposure of HK and spleen leukocytes to 300 and 500 mOsm up to 24 h failed to inhibit cell viability. When comparing RB activities at the 2 osmolalities without adding serum, HK and spleen leukocytes cultured at 500 mOsm osmolality for 4, 8, and 24 h were found to have higher activities than those cultured at 300 mOsm osmolality. RB activities of HK and spleen leukocytes showed significant decreases in both 300 and 500 mOsm incubated cells after adding serum, and the decrease was more distinct at an osmolality of 500 mOsm. These results imply that the addition of serum might cause a suppressive effect on cellular parameters rather than improving the effects of cytokines on cellular parameters. http://zoolstud.sinica.edu.tw/Journals/51.8/1290.pdf

Key words: Respiratory burst activity, Stress-activated serum, Freshwater (FW)-exposed tilapia, Hyperosmolality.

Animals have very complicated and delicate responses to stress that protect against environmental perturbations, which may be disadvantageous to their physiology, psychology, growth, and breeding. Fish change their feeding activity from diurnal to nocturnal when exposed to this type of stress combined with the relatively bright illumination during daytime (Ferter and Meyer-Rochow 2010). Gills and kidneys are the most important organs for osmoregulation in teleosts, and fundamental transporters responsible for ion movements across gill and kidney epithelia have been widely studied (Marshall and Grosell 2006, Marshall 2002). Short-term starvation (stress) dampens trypsin activity and the trypsin/chymotrypsin ratio in relation to the growth of tilapia (Chan et al. 2008). Adverse environmental situations may acutely or chronically disrupt the health of fish, by altering some of their biochemical parameters and suppressing their innate and adaptive immune responses (Miller et al. 2002). Changes in salinity can naturally occur as a consequence of rainwater diluting seawater (SW), mixing of estuarine waters, or the ingress of SW into normally freshwater (FW) areas. Many fish species are capable of withstanding large osmotic changes (Bowden 2008). Maintenance of the water balance in fish (osmoregulation) can

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be disrupted by changes in the metabolism of minerals. In such circumstances, FW fish absorb excessive amounts of water from the environment (over-hydrate), while SW fish lose water to the environment (dehydrate); such disruptions increase energy requirements for osmoregulation (Rottmann et al. 1992). Tilapia are a commonly used animal model to investigate osmoregulatory mechanisms (Sardella and Brauner 2008), as this species can live and thrive in a wide range of salinities from FW to salt concentrations higher than SW (hypersaline) (Iwama 1997), and can survive in SW up to 120 parts of per thousand (ppt) (Stickney 1986). In our previous study, *Oreochromis mossambicus* survived when it was directly transferred to 25 ppt SW (Weng et al. 2002). Other studies showed that severe changes (to > 35 ppt SW) in salinity resulted in death, while contradictorily, a gradual transfer from low to high salinities caused fish to thrive even in 120 ppt SW (Yang et al. 2009). Another report showed that the short-term (4-d) response of *O. mossambicus* to different water salinities increased their oxygen consumption rates in SW compared to both FW and isotonic salinity (Morgan et al. 1997). Heat shock protein (HSP)70 and HSP90 may be involved in regulation of the tilapia brain faced with acute osmotic stress and subsequently facilitate adaptation to an SW environment (Yang et al. 2009).

Several reactive oxygen species (ROS) are produced by fish phagocytes during respiratory bursts (RBs). Once bacteria or fungi are engulfed by leucocytes, a host's NADPH-oxidase is activated, which in turn increases oxygen consumption and subsequently produces ROS such as superoxide anions, hydrogen peroxide, hydroxyl radicals, and singlet oxygen (Roch 1999). This release of superoxide anions is known as an RB, and its derivates are bactericidal (Secombes and Fletcher 1992). One major factor responsible for fish becoming susceptible to disease during acute and chronic stresses is the alteration in both the number and composition of circulating leukocytes (Barton and Iwama 1991). Stress also affects the phagocytic and/or RB activities of leukocytes of the spleen, head kidneys (HKs), and plasma (Thompson et al. 1993, Pulsford et al. 1994). A study of brown trout (*Salmo trutta*) transferred from FW to SW showed an increase in the phagocytic activity of HK leukocytes (Marc et al. 1995). Similarly, when tilapia were directly transferred from FW to SW, we observed increased plasma lysozyme, alternative complement protein, phagocytic, and RB activities (Jiang et al. 2008). In another study, we found that the addition of serum from SW-exposed tilapia decreased phagocytic activities of HK and spleen leukocytes when cultured in 300 and 500 mOsm (Kumar et al. 2009). Similarly, HK and spleen leukocytes of rainbow trout moved from FW to SW showed increased RB activities (Taylor 2007). In order to better understand the Mozambique tilapia's osmoregulatory ability, the present study attempted to (i) determine the proliferation efficiency of HK and spleen leukocytes at osmolalities of 300 and 500 mOsm; (ii) isolate serum from tilapia exposed to FW and 25 ppt SW adding into cells for 2 h; and (iii) further characterize the role of stress-activated serum in regulating RB activities of HK and spleen leukocytes by culturing them in media with osmolalities of 300 and 500 mOsm for various times.

MATERIALS AND METHODS

Fish

Tilapia (*Oreochromis mossambicus*) of approximately 8-10 cm in total length measuring (10.44 ± 0.4 g body weight) were obtained from the Mariculture Research Center of the Fisheries Research Institute (Keelung, Taiwan). They were maintained at the National Dong Hwa Univ. Animal Laboratory in an FW recirculation tank with a 12-h light: 12-h dark photoperiod at 24-28°C and fed daily with a commercial diet (Tung-Li Feed Industrial, Pingtung, Taiwan). Fish were reared in circular FW tanks supplied with a filter and an aeration system. All experiments were performed according to the *Guide for the Care and Use of Laboratory Animals* of National Dong-Hwa Univ.

Prior to the experiments, fish were acclimated to laboratory conditions for more than 1 mo. Both male and female tilapia, ranging 35-65 g (mean ± standard deviation (S.D.), 50 ± 20 g) with no significant differences among treatments, were used in these experiments. For the hyperosmolality (SW) challenge tests, one time point (2 h) was used, and 4 fish per exposure time were maintained in individual tanks. Two additional tanks of fish were kept in FW as controls.

Serum collection

Five fish per group were carefully netted and then anesthetized with a 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 blood was transferred to 1.5 mL centrifuge tubes and stored on ice. The blood sample was stored overnight at -20°C and spun down at 400 xg for 10 min. The separated serum was stored in sterile Eppendorf tubes at -80°C until being used.

Cell proliferation assay

Cell proliferation was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma) (Mosman 1983). To measure the cell proliferation of HK and spleen leukocytes of FW-exposed tilapia, leukocytes (2×10^5) were incubated in 96-well plates with 10% fetal bovine serum (FBS) medium and then treated with osmolalities of 300 and 500 mOsm for 4, 8, and 24 h. The osmolality of the media was adjusted by adding appropriate amount of 0.5% NaCl. After incubation, the medium was replaced with 20 μL of MTT reagent (5 mg/mL) and then incubated at 24ºC for 4 h. Afterwards, the medium was aspirated, 200 μL of dimethyl sulfoxide was added to each well, and the absorbance was measured at 570 nm.

Preparations of HK and spleen leukocytes for assaying phagocytic activities

To examine the effects of extracellular osmolality, serum, spleens, and HKs were collected. Leukocyte preparations from the spleen and HKs were described in our previous study (Jiang et al. 2008). Briefly, cell suspensions were prepared in RPMI-1640 (Gibco, Grand I., NY, USA) medium by grinding the head kidney and spleen with ground glass and filtering them through a nylon mesh. Leukocytes were separated on a 51% Percoll gradient (Sigma) in phosphate-buffered saline (PBS) (Gibco). Cells were collected at the interface layer, washed twice, and then resuspended in RPMI-1640 medium. Subsequently, cell suspensions were centrifuged at 500 xg at room temperature (RT) for 10 min. HK and spleen leukocytes (5×10^5 cells) were individually pre-incubated in a 96-well plate containing 100 μL of RPMI 1640 medium with Earle's salt (Gibco). This medium was buffered with 25 mM of 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) and 18 mM of sodium bicarbonate ($NaHCO_3$) (pH 7.4), supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL), and 10% FBS (Sigma). Cultured cells were incubated in medium with an osmolality of 300 or 500 mOsm containing serum of tilapia exposed to FW or SW for 4, 8, and 24 h at 28°C. After incubation, the RB activities of immune cells were measured.

RB assay

RB activities of HK and spleen leukocytes from tilapia were studied by flow cytometry as previously described (Craig et al. 2005). Phagocytes were suspended in RPMI-1640 medium (Gibco) at a concentration of 5×10^5 cells/mL. Samples (100 μL) of HK and spleen leukocyte suspensions were mixed with 0.05 M of dichlorofluorescin diacetate (DCFH-DA) (Sigma) in the dark on a shaker platform (55 rpm/min) at RT for 25 min. In the presence of ROS, DCFH-DA is oxidized to dichlorofluorescein (DCF), which can be detected by flow cytometry (Cytomics™ FC500, Beckman, Fullerton, CA, USA) and provides a quantitative assessment of RBs in individual cells. No stimulatory agent was added to analyze the RB activity. After incubation, cells were washed twice with cold PBS and resuspended in 500 μL of cold PBS for the flow cytometric analysis, which was performed within 30 min. Cells with a fluorescence intensity of greater than background were considered positive, indicating that RBs had occurred. The percentage of RBs was defined as the percentage of cells which had undergone RBs among the total cell population. The RB activity per cell was determined from the mean fluorescence intensity of cells.

Statistical analysis

Experimental data are presented as the mean ± S.D. Control and treatment groups were compared by a one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) *t*-test (SAS 2004). Differences among treatment groups at different times were analyzed using the LSD test. * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) indicate significant differences from the control group.

RESULTS

Effect of salinity on cell proliferation

The effects of salinity on the cell proliferation

of HK and spleen leukocytes were examined with an MTT assay. Experimental data indicated that HK and spleen leukocytes exhibited no significant reductions in cell viability after exposure to 300 or 500 mOsm osmolality for various time points (Fig. 1A, B).

In vitro RB activities of HK leukocytes

The effect of salinity on RBs was determined using HK leukocytes with 3 different treatment groups of (a) a control without serum addition, (b) the addition of 25% serum from tilapia exposed to FW, and (c) the addition of 25% serum from tilapia exposed to 25 ppt SW for 4, 8, and 24 h as compared to the *in vivo* control. RB activities of HK leukocytes in the group with serum from SW-exposed tilapia exhibited a significant difference in osmolalities of 300 and 500 mOsm (Fig. 2). When RB activities were compared between media with osmolalities of 300 and 500 mOsm, HK leukocytes cultured in medium with an osmolality of 500 mOsm showed higher RB activities than those cultured in medium with an osmolality of 300 mOsm in each corresponding group (Fig. 3).

In vitro RB activities of spleen leukocytes

Treatment groups used for measuring RB activities of spleen leukocytes were similar to those for HK leukocytes. Significant differences were seen in the groups exposed to 300 and 500 mOsm osmolalities. RB activities of splenocytes with addition of serum from the SW group significantly differed between the 300- and 500-mOsm-osmolality groups. Spleen leukocytes

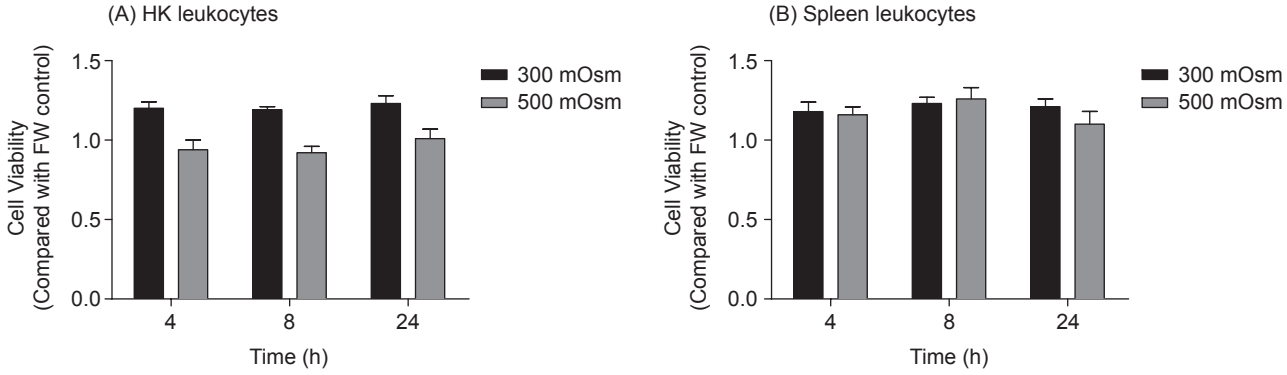

Fig. 1. *In vitro* cell viability of (A) HK leukocytes and (B) Spleen leukocytes. The leukocytes were cultured at 300 and 500 mOsm for 4, 8 or 24 h, respectively. The cell viability was measured using MTT assay and data were compared with cells incubated with FW for 0 h. Data are expressed as mean ± S.D. (pool sample, $n = 4$).

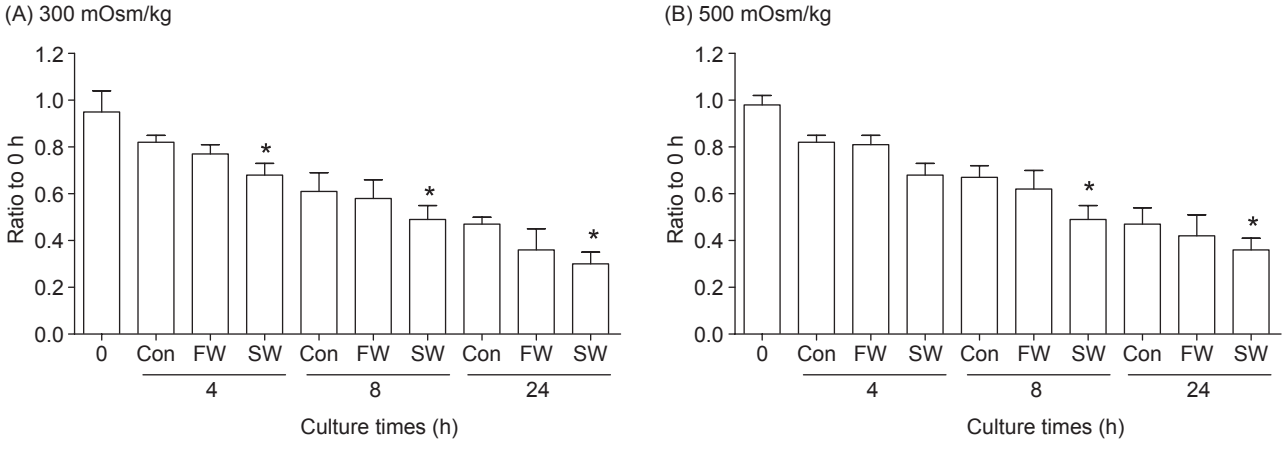

Fig. 2. Respiratory burst activity of head kidney leukocytes in (A) 300 mOsm/kg, and (B) 500 mOsm/kg osmolality media after cultured for 4, 8 or 24 h. Con: control, non-serum added; FW: added 25 % serum of tilapia in fresh water; SW: added 25 % serum of tilapia in 25 ppt sea water. Data are expressed as mean ± S.D. (pool sample, $n = 8$). *($p < 0.05$) indicates statistically significant differences as compared with the control at corresponding time.

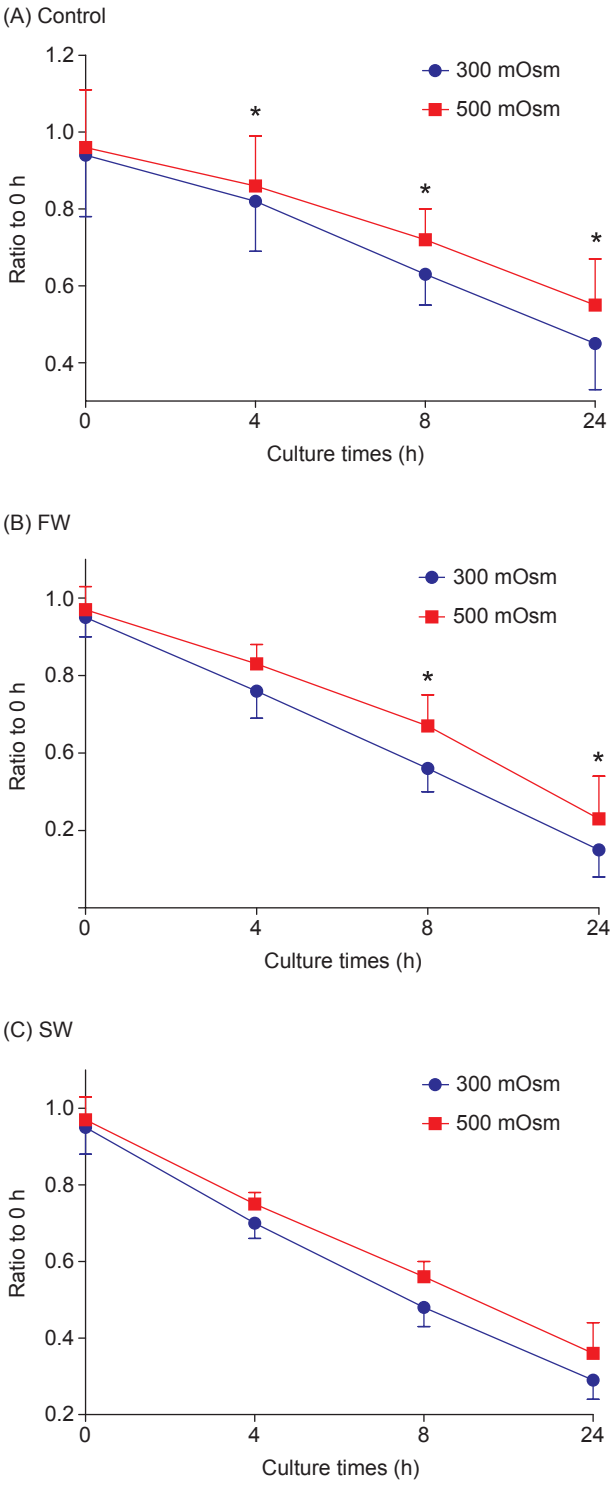

Fig. 3. *In vitro* HK respiratory burst compared between 300 and 500 mOsm/kg osmolality media after cultured for 4, 8, or 24 h. (A) Con: control, non-serum added; (B) FW: added 25 μL serum of tilapia in FW; and (C) SW: added 25 μL serum of tilapia in 25 ppt SW. Data are expressed as mean ± S.D. (pool sample, n = 6). *($p < 0.05$) indicates statistically significant differences compared with the control.

cultured in medium with an osmolality of 500 mOsm for 4 h showed increased RB activity in the control group compared to those cultured in media with serum from the FW and SW groups (Fig. 4). When RBs were compared between the groups with osmolalities of 300 and 500 mOsm, spleen leukocytes cultured at 300 mOsm showed decreased RC activity as compared to those cultured in 500-mOsm medium in each corresponding group (Fig. 5).

DISCUSSION

Environment salinities influence the distribution and survival of aquatic and marine

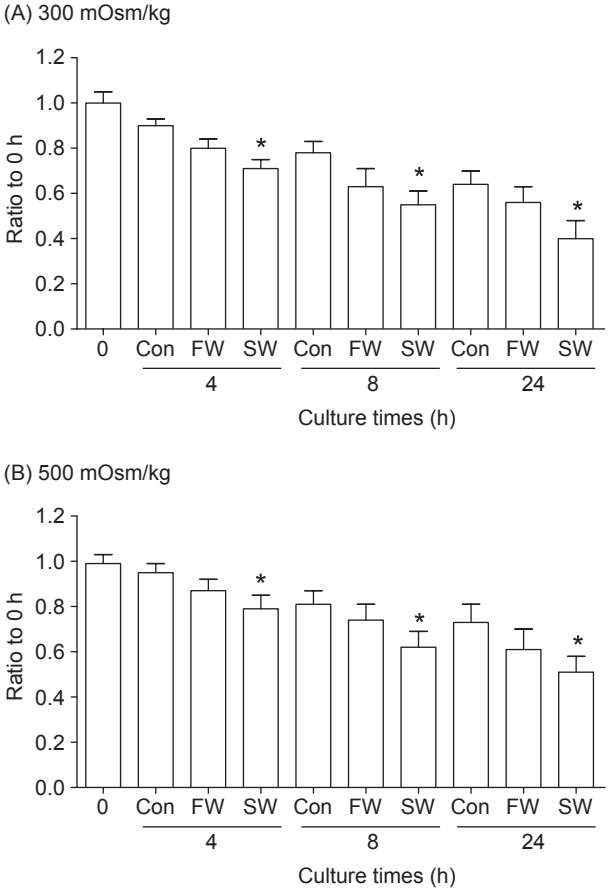

Fig. 4. Respiratory burst activity of spleen leukocytes in (A) 300 mOsm/kg and (B) 500 mOsm/kg osmolality media after cultured for 4, 8 or 24 h. Con: control, non-serum added; FW: added 25 % serum of tilapia in fresh water; SW: added 25 % serum of tilapia in 25 ppt sea water. Data are expressed as mean ± S.D. (pool sample, n = 8). *($p < 0.05$) indicates statistically significant differences as compared with the control at corresponding time.

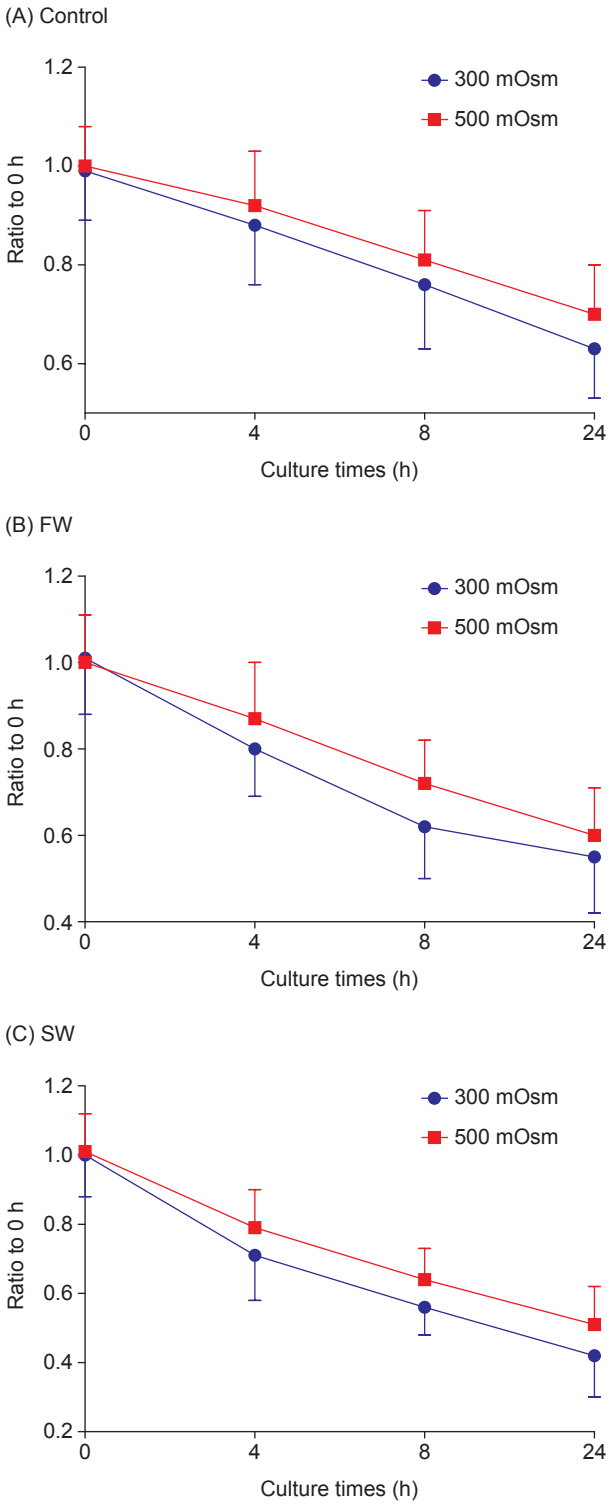

Fig. 5. *In vitro* spleen respiratory burst compared between 300 and 500 mOsm/kg osmolality media after cultured for 4, 8, or 24 h. (A) Con: control, non-serum added; (B) FW: added 25 μL serum of tilapia in FW; and (C) SW: added 25 μL serum of tilapia in 25 ppt SW. Data are expressed as mean ± S.D. (pool sample, n = 6).

organisms (Bodinier et al. 2009). Stresses due to salinity changes were reported to increase the values of some blood components (Vijyan et al. 1997). Such changes could affect both oxygen transport in the blood and transfer across the gills (Akinrotimi et al. 2012). RBs are a crucial reaction that occurs in phagocytes to degrade internalized particles and bacteria, and it is influenced by environmental factors such as toxicants, temperature, and salinity. Salinity is a major stress factor, and increased salinities can increase phagocytosis and RBs (Bowden 2008). Thus, in the present study, we first exposed tilapia to FW or 25-ppt SW and then isolated the blood serum. From our proteomics analysis we found that acute exposure of tilapia to SW resulted in increased expression of the complement C3 protein (Kumar et al. 2009).

Salinity adaptation by teleosts involves osmoregulation and affects tissues like the HKs and spleen (Bodinier et al. 2009, Kumar et al. 2009). Tilapia can tolerate 170%-180% SW, or around 58.65-63 ppt, with significant increases in plasma osmolality (Kültz et al. 1992, Uchida et al. 2000). Similarly, when Parr (Atlantic Salmon, *Salmo salar* L.) smolt were transferred to seawater, significant changes in peripheral blood and HK leukocytes were observed (Manning and Nakanishi 1996). Plasma osmolalities significantly increased with increases in environmental salinity and temperature when tilapia were exposed to FW, SW, and 200% SW at 20, 28, and 35°C (Fiess et al. 2007). Thus, we isolated HK and spleen leukocytes from tilapia exposed to osmolalities of 300 and 500 mOsm and tested the cell viability at 3 different time points. Compared to HK leukocytes exposed to 300 mOsm, exposure to 500 mOsm decreased the cell viability, but it increased during 24 h of exposure. Contrarily when spleen leukocytes were incubated in media at 300 and 500 mOsm, they showed no alterations in cell viability.

Numerous studies demonstrated that exposure of fish to environmental contaminants modulated fish phagocytic ROS production (Zelikoff 1994, Zelikoff et al. 1995, Roszell and Anderson 1996). Decreases in RBs and phagocytic activity occurred when leukocytes of seabream *Sparus aurata* were incubated with cortisol (Esteban et al. 2004). In the present study, we analyzed the effect of stress-activated serum on RB activity during a change in environment salinity. Compared to the 0-h control, after serum addition, RB activity decreased with time. However, when

comparing RBs between the 300 and 500 mOsm groups, RB activity of 500 mOsm-exposed HK leukocytes increased. In the spleen leukocyte group, control cells cultured for 4 h in 300-mOsm medium showed increased RB activity compared to other time points, while control cells cultured in 500 mOsm medium showed higher RB activity than those of the serum-added FW and SW groups. Thus, significant changes were observed in serum-exposed spleen leukocytes when incubated in media at 300 and 500 mOsm. Both HK and spleen leukocytes incubated in 500 mOsm medium showed increased RB activity compared to cells cultured in 300 mOsm medium. Transfer of fish from conditions of hypo-osmolarity to hyperosmolality significantly enhances plasma lysozyme, alternative complement pathway (ACP), phagocytic, and RB activities (Jiang et al. 2008). In a recent study, we found that increased RB activity decreased after the addition of serum.

In conclusion, groups to which serum was added showed decreased RB activity compared to the FW group without serum addition. These data are consistent with our previous study (Kumar et al. 2009), which showed that the addition of serum decreased the phagocytic ability of HK and spleen leukocytes upon exposure to media at 300 and 500 mOsm. Thus, the addition of serum might cause suppressive effects on cellular parameters rather than improving cytokines' effects. With reference to our previous study, that work proposes that proteins present in SW-exposed tilapia serum, including semaphorin, complement C3, Mg^{2+}-dependent neutral sphingomyelinase, and caspase 3, might play central roles in decreasing RB activity.

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