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SHORT COMMUNICATION

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The extent to which lipopolysaccharide modulates oxidative stress response in *Mugil cephalus* juveniles

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Aquaculture is incessantly infected with various types of micro-organisms from different origin, including bacteria. Immunostimulants are profusely and efficiently used as feed additives in aquaculture (Dawood, Koshio, Abdel-Daim, & Van Doan, 2019; Dawood, Koshio, & Esteban, 2018). Lipopolysaccharide (LPS) is a product of gram-negative bacteria cell wall, act as a potent immunomodulator of innate immune response (Bishop, 2005; Raetz & Whitfield, 2002), which is characterized by a production of proinflammatory cytokines, and reactive oxygen species (ROS) (Abo-Al-Ela, 2018b; West & Heagy, 2002). Once the production of ROS exceeds the cellular antioxidant capacity (Agnisola, 2005; Palumbo, 2005), it leads to oxidative stress (Feng, Zhang, Zheng, Xie, & Ma, 2008; Halliwell & Whiteman, 2004) that results in severe cellular injury (i.e. lipid, protein and DNA destructions) (Abo-Al-Ela, 2019; Abo-Al-Ela, El-Nahas, Mahmoud, & Ibrahim, 2017b; Mujahid et al., 2007). This injury shifts cellular net charge and disturbs cellular osmotic pressure that in turn result in swelling of cell and eventually cell death (van Hoorn et al., 2001). The deleterious effect of excessive ROS is balanced by many cellular defence activities, including antioxidant defence mechanisms (Paital & Chainy, 2010). The antioxidant defence system works to keep ROS at lower levels (Paital & Chainy, 2010) by improving the efficiency of the mitochondrial electron chain (Fazio, Piccione, Saoca, Caputo, & Cecchini, 2015). The main molecules of the antioxidant defence network are three antioxidant enzymes: superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), which they inhibit ROS production by eliminating their precursors (Surai, 2016).

Fish, including grey mullet, *Mugil cephalus* have an ecological and a socio-economic relevance in many countries, including Egypt (Abo-Al-Ela, 2018a; Abo-Al-Ela, El-Nahas, Mahmoud, & Ibrahim, 2017a). Fish are highly susceptible to oxidative stress compared with other species due to their habitats (Birnie-Gauvin, Costantini, Cooke, & Willmore, 2017; Francesco Fazio et al., 2013). The aquatic environment receives a daily substantial amount of environmental pollutants that may cause oxidative stress in aquatic organisms via free radical and ROS-involved pathways (Valavanidis, Vlahogianni, Dassenakis, & Scoullos, 2006). Mullet is highly sensitive to environmental changes that could affect its physiological functions, such as blood fluidity and oxygen transport (Lee & Pan, 2003). In addition, mullet is a bottom feeder, which this feeding habit increases the risk of getting stressed through exposure to deleterious materials, such as heavy metals (Fazio et al., 2015; Gorbi, Baldini, & Regoli, 2005). Stocking density in fish rearing was found to modulate oxidative status (Dawood, Shukry, et al., 2019). Although many studies have demonstrated the physiological response of grey mullet to oxidative stress of environmental pollutions (Akbary, 2018; Lee & Pan, 2003; Min, Ahn,

& Kang, 2016), there is a lack of knowledge regarding its response following microbial infection. Thus, the aim of the current study was to examine the oxidative stress response following injection of LPS with different doses in grey mullet. This was achieved by measuring the antioxidant capacity of SOD and CAT enzymes, leucocyte count as well as heterophils/lymphocytes (H/L) ratio.

A total of 240 healthy juvenile grey mullet, *Mugil cephalus* with an average body weight 9 ± 0.5 g were obtained from a commercial fish farm, Kafrelsheikh governorate, Egypt. Fish were randomly allotted into 4 treatment groups (60 fish each which allocated into 2 replicates). Fish were kept in glass aquaria (90 × 50 × 35 cm) at a temperature 28 ± 2°C and a pH 7.7 ± 0.2 and supplied with continuous aeration. The glass aquaria were cleaned daily, and about 50% of water was replaced with clean dechlorinated tap water. Fish were fed at 3% of live body weight per day.

Figure 1 shows the experimental design of the current study. Briefly, following the acclimation period (2 weeks), each group of fish were intraperitoneally injected with LPS and normal saline. First group (1st) was injected with normal saline (control group), while, 2nd, 3rd and 4th groups were injected with LPS at concentrations of 1, 10 and 100 µg/kg fish weight respectively.

Samples were collected at five different time postinjection (6, 12, 24 and 48 hr and one week postinjection). Fish (n = 6) from each treatment group (3 fish/replicate) were randomly selected. Blood samples were collected by puncturing the caudal vein into tubes contain anticoagulant (EDTA). After that, fish were carefully dissected and liver samples were taken from each fish, and subsequently kept at −20°C for measuring the antioxidant capacities.

A drop of blood was smeared on glass slide, left to dry and fixed by methyl alcohol for 3–5 min, then stained by Giemsa for 20 min according to Houwen (2002). Then, slides were rinsed with water, covered by filter papers and left to dry. Blood films were examined with a light microscopy using an oil immersion objective. The percentage of each type of blood cells were calculated.

Liver homogenates were prepared by homogenization in sterile cold potassium phosphate buffer (pH 7). Clear supernatant was obtained from homogenates via spinning at 4,020 × g for 15 min at 4°C; then, the supernatant was stored at −20°C for the further parameters analysis. Antioxidant enzymes activities (U/g tissue) of superoxide dismutase (SOD) and catalase (CAT) were determined using a commercial kit (Biodiagnostic - SD2521, Egypt), and a UV-VIS spectrophotometer according to the method described by (Aebi, 1984). SOD activity was measured spectrophotometrically at 560 nm over 5 min. Regarding CAT, in brief, it reacts with a known amount of H_2O_2 for 1 min; and then a CAT inhibitor used to stop the reaction. Then, the remaining H_2O_2 reacts with 4-aminophenazone and 3,5-Dichloro-2-hydroxybenzene sulphonic acid to form a chromophore in the presence of peroxidase. Finally, the absorbance can be measured at 240 nm over 3 min, in which colour intensity is inversely proportional to the levels of CAT.

Data were analysed using GraphPad Prism 6 software (GraphPrism Software, Inc.). Two-way ANOVA followed by Tukey's multiple comparison was run to examine statistically significant differences at $p < .05$ according to the following model: $Yij = \mu + Li + Tj + (TS)ij + \varepsilon ij$, where Yij is the measured dependent variables, µ is the overall mean, Li is the effect of different LPS doses, Tj is the effect of time factor, (LT)ij is the interaction between i and j, and εij is the random error. The results were stated as mean ± *SE* and were considered significant at $p < .05$.

Lipopolysaccharide modulated the differential leucocyte count at different time interval postinjection (Table S1). LPS significantly changed the lymphocytes count at different doses and times postinjection without interaction between LPS doses and time (p = .002 for LPS; p = .015 for time; $p > .05$ for interaction). After 6 hr postinjection, LPS at doses of 1 and 100 µg significantly increased the lymphocytic counts compared with normal saline and 10 µg LPS ($p < .05$). On the other side, with the time progress after the injection, time factor was significantly associated with a marked decrease

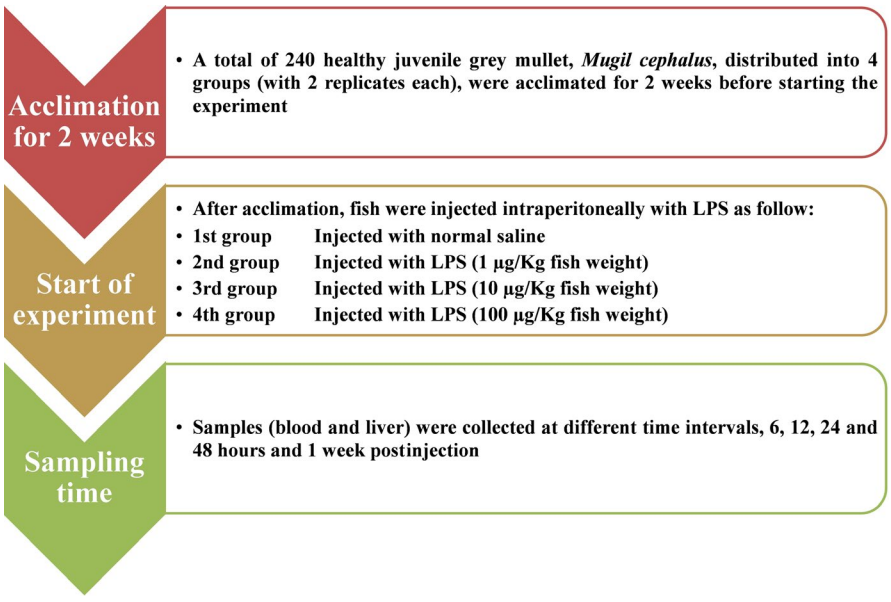

FIGURE 1 The experimental design of the current study

in the lymphocytic count especially after one week of injection ($p < .05$). Different doses of LPS and time of sampling significantly changed the heterophils count without interaction ($p = .007$ for LPS; $p = .025$ time; $p > .05$ for interaction). After 6 hr, injecting LPS at doses of 1, 10 and 100 μg/kg fish weight significantly lowered the heterophils count compared with normal saline ($p < .05$). However, at 12, 24 and 48 hr, the doses of 1 and 100 μg distinctly decreased the heterophils count compared with normal saline and 10 μg of LPS ($p < .05$). After one week of LPS, stimulation did not change the heterophils count ($p > .05$). The different doses of LPS as well as the time factor did not had any effect on monocyte, eosinophil and basophil counts ($p > .05$).

Lipopolysaccharide markedly altered the heterophils/lymphocytes (H/L) ratio (Figure 2) in all times of sampling without interaction. LPS (except for the dose 10 μg after 12 hr) significantly reduced the H/L ratio at 6 and 12 hr compared with normal saline ($p < .05$). After 24 hr postinjection, 100 μg LPS was the only dose that significantly lowered H/L ratio among the treatments ($p < .05$). While, after 48 hr following injection, there was a significant reduction in the H/L ratio in all dose of LPS compared with normal saline ($p < .05$). After one week, LPS did not change the H/L ratio ($p > .05$). In addition, at dose 1 μg, there was a marked increase in the H/L ratio after one week of injection compared with the H/L ratio at 6 and 12 hr postinjection ($p < .05$). Additionally, after one week of injection, there was a significant increase in the H/L ratio in the case of 10 μg LPS compared with 6 hr postinjection ($p < .05$). Likewise, after one week, 100 μg LPS-injected fish showed a distinct increase in the H/L ratio compared with 6, 12 and 24 hr ($p < .05$).

Figure 3 shows the effect of different doses of LPS, and time effect postinjection on the CAT activities in the liver. Different LPS concentrations and time intervals postinjection significantly changed the CAT activities with a marked interaction. After 6 hr, there were no changes in the CAT activities ($p > .05$). However, after 12, 24 and 48 hr, there were significant reductions in CAT activities in doses of 10 and 100 μg compared with normal saline and 1 μg LPS ($p < .05$). While after one week postinjection, there were no changes in the CAT activities ($p > .05$). Moreover, with the passage of time and at the level of the same LPS dose, there was an alteration in the CAT activities. Additionally, at the dose 1 μg, the highest activity of CAT was found at 24 hr ($p < .05$). However, the activity of CAT was

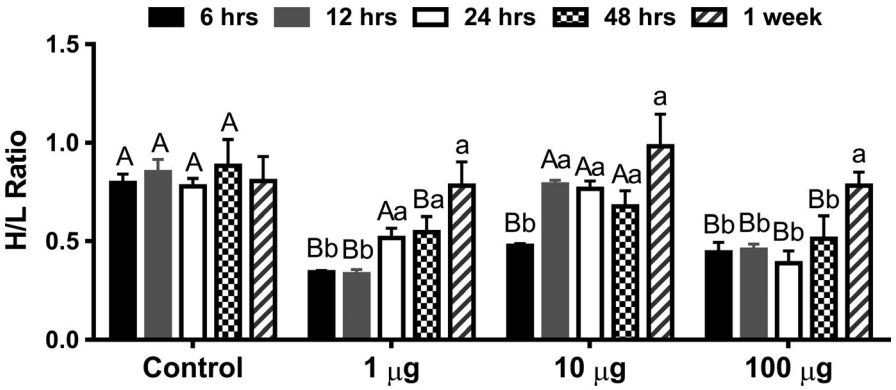

FIGURE 2 The effects of different doses of LPS and time intervals on the heterophils/lymphocytes (H/L) ratio. The results are presented as means ± *SE*. Lowercase letters indicate significant differences among different treatments at the same time. While, uppercase letters indicate significant differences between different times at the same dose. The *p*-values of two-way ANOVA were as follows: LPS, <0.0001; time, <0.0001; LPS × time, 0.0054

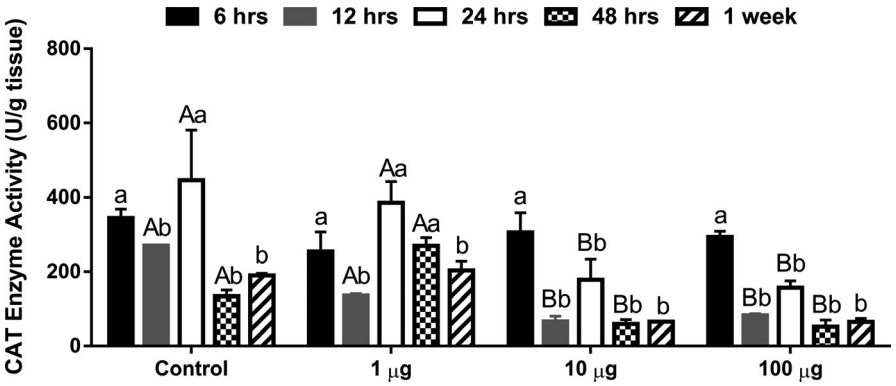

FIGURE 3 The effects of different doses of LPS and time intervals on the catalase (CAT) enzyme activities (U/g tissue) in the liver of grey mullet, *Mugil cephalus*. The results are presented as means ± *SE*. Lowercase letters indicate significant differences among different treatments at the same time. While, uppercase letters indicate significant differences between different times at the same dose. The *p*-values of two-way ANOVA were as follows: LPS, <0.0001; time, <0.0001; LPS × time, 0.0054

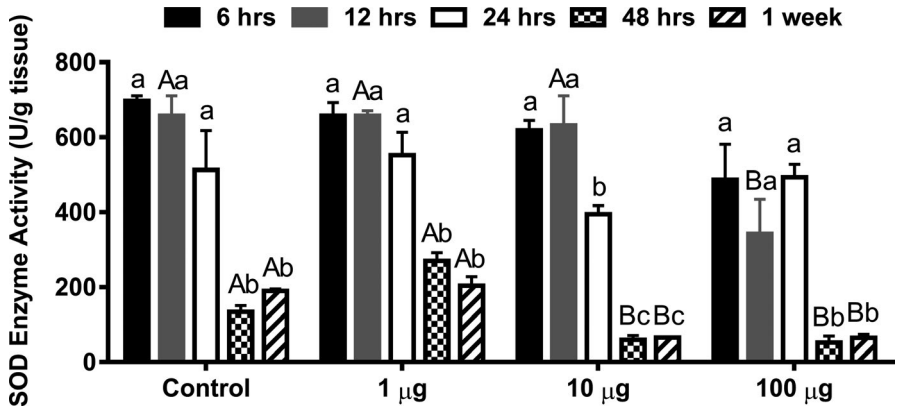

FIGURE 4 The effects of different LPS doses and exposure times on the sodium oxide dismutase (SOD) enzyme activities (U/g tissue) in the liver of grey mullet, *Mugil cephalus*. The results are presented as means ± *SE*. Lowercase letters indicate significant differences among different treatments at the same time. While, uppercase letters indicate significant differences between different times at the same dose. The *p*-values of two-way ANOVA were as follows: LPS, 0.0035; time, 0.023; LPS × time, 0.0119

significantly declined at 48 hr, and the decrease was continued till one week postinjection ($p < .05$). Also, in the case of 10 and 100 μg, a significant reduction in the CAT activities was noticed with the progress of time ($p < .05$).

Testing the effect of different LPS doses and time intervals postinjection on the hepatic SOD activities (Figure 4) indicated a significance of both factors with a significant interaction. After 6 hr postinjection, there was no changes in the SOD activities ($p > .05$). While, after 12 hr postinjection, a significant reduction in the level of SOD was noticed in dose 100 μg compared with other treatments ($p < .05$). However, this effect was disappeared after 24 hr postinjection, hence, the SOD activities did not show any changes in all groups ($p > .05$). Nevertheless, at 48 hr and one week postinjection, significant decreases of SOD activity were detected in doses of 10 and 100 μg compared with other treatments ($p < .05$).

Furthermore, there was a change in the SOD activities within the same treatment. In the case of normal saline and 1 μg LPS, significant declines in the level of SOD were recorded at 48 hr and one week postinjection ($p < .05$). Also, the SOD activities were significantly dropped with the passage of time in the dose of 10 and 100 μg. Where at 24 and 48 hr, and one week, it showed a significant low level compared with 6 and 12 hr ($p < .05$).

Inflammation and oxidative stress are strictly correlated. Oxidative stress is associated with excessive production of ROS, which may exceed the antioxidant capacity (Jiang et al., 2017). Bacterial infection causes an inflammatory response, and is usually associated with secretion of many bacterial endotoxins such as LPS, which is a main component of the gram-negative bacterial wall that is able to trigger and accelerate oxidative stress in mammals and fish (Bai & Gu, 2017; Bich Hang, Nguyen, & Kestemont, 2016; Li et al., 2016). In mice, it has been shown that LPS induces oxidative stress as a result of decreasing the antioxidative enzymes activity, including SOD, CAT, glutathione (GSH) and GPx (Jiang et al., 2016). However, there are no evidences regarding the same activities on fish response, particularly grey mullet.

The assessment of stress response can be done by measuring the levels of stress indicator hormones, such as plasma glucocorticoids, including corticosterone (Davis, Maney, & Maerz, 2008). However, measuring these hormones was clearly associated with many drawbacks. For example, their levels upsurge rapidly following animal handling, result in difficulties to obtain baseline measurements or precise results (Romero & Reed, 2005). Therefore, it is necessary to use alternative approaches, such as assessing other haematological parameters, for example, the relative counts of white blood cell (WBC) (Davis et al., 2008). Any particular changes in leucocyte count are related to stress hormone levels. Besides, this approach provides some advantages over direct assessment of the glucocorticoid in plasma, which leucocyte population takes a relatively much time to be changed, as well as it is relatively inexpensive (Davis et al., 2008). In the current study, increasing the dose of LPS alongside with the passage of time postinjection was associated with the modulation of the differential leucocyte counts that resulted in increases of the H/L ratio. The increases in H/L ratio and leucocytes may be an indicator for a stress from the LPS; in particular, this effect was increased with increasing the dose of LPS, and continued with the progress of time (Dhabhar, Miller, McEwen, & Spencer, 1996). In addition, the increased H/L ratio may be resulted from increasing transmigration of lymphocytes from the circulation to tissues, such as spleen and lymph nodes, as well as heterophil influxes to circulation as a result of the increases in stress hormones (Dhabhar, 2002).

Antioxidant enzymes are good biomarkers for oxidative stress; they play a crucial role in competing the damaging effects of oxidative stress that results from over production of ROS (Kurhalyuk & Tkachenko, 2011). Where they are key components that protect cells, and attempts to save species' survivability during oxidative stress (Lushchak & Bagnyukova, 2006). In the present investigation, it was found that LPS treatment was associated with a marked reduction in the hepatic CAT activities in a dose-dependent manner especially at doses of 10 and 100 μg. This effect was also seen over the time after the LPS injection. Presumably, the reduction in the CAT activities was resulted from the inhibitory action of the

overproduced ROS, suggesting an increase in the stress status of grey mullet juveniles (Pigeolet et al., 1990). This finding was in accordance with Abdel-Daim et al. (2019), Chitra and Maiby (2014), Faheem, Jahan, and Lone (2016) and Wu, Xu, Shen, Qiu, and Yang (2011), which they reported a reduction in the hepatic CAT activity in tilapia exposed to sublethal doses of bisphenol-A and cadmium, and intoxication with zinc oxide nanoparticles. Other possible explanation, it may be from exhaustion or damage of the hepatic tissue (Abdel-Daim et al., 2019; Faheem et al., 2016). To further explain, low doses of LPS or even after few hours postinjection in high doses, fish might be able to accommodate or curb the stress especially after 24 hr, however, when time goes by, fish become unable to overcome this (Faheem et al., 2016).

Similar records were detected in the SOD activities. Possibly, the decline in the SOD activities is associated with ROS-induced hepatic peroxidation, and a damage in the hepatic tissue (Velkova-Jordanoska, Kostoski, & Jordanoska, 2008) or extra-generation of ROS resulted from lipid peroxidation (Akbary, Sartipi Yarahmadi, & Jahanbakhshi, 2018). Similar results were obtained by Akbary et al. (2018), who documented a reduction in the SOD activities in grey mullet exposed to copper oxide.

Our results showed a significant alteration in antioxidative responses to LPS in the grey mullet. There was a distinct variation in the H/L ratio, and antioxidant enzymes activities, CAT and SOD a dose- and time-dependent manners. Future work on other inflammatory responses, such as fractionation of serum proteins or relative gene expression analysis could provide clearer image about the effect of LPS on the antioxidant capabilities and immune response in grey mullet.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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