The modulation role of serotonin in Pacific oyster

Crassostrea gigas in response to air exposure

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Abstract:

Serotonin, also known as 5-hydroxytryptamine (5-HT), is a critical neurotransmitter in the neuroendocrine-immune regulatory network and involved in regulation of the stress response in vertebrates and invertebrates. In the present study, serotonin was found to be widely distributed in the tissues of Pacific oyster *Crassostrea gigas*, including haemolymph, gonad, visceral ganglion, mantle, gill, labial palps and hepatopancreas, and its concentration increased significantly in haemolymph and mantle after the oysters were exposed to air for 1 d. The apoptosis rate of haemocytes was significantly declined after the oysters received an injection of extra serotonin, while the activity of superoxide dismutase (SOD) in haemolymph

increased significantly. After the stimulation of serotonin during air exposure, the apoptosis rate of oyster haemocytes and the concentration of H₂O₂ in haemolymph were significantly decreased, while the SOD activity was significantly elevated. Furthermore, the survival rate of oysters from 4th to 6th d after injection of serotonin was higher than that of FSSW group and air exposure group. The results clearly indicated that serotonin could modulate apoptotic effect and redox during air exposure to protect oysters from stress.

Keywords: *Crassostrea gigas*; Environmental stress; Air exposure; Serotonin; Apoptosis; Redox

Introduction

Serotonin (5-hydroxytryptamine, 5-HT), one of the earliest-identified neurotransmitters in serotonergic neuroendocrine system [1], is derived from the amino acid tryptophan and synthesized by the rate-limiting enzyme tryptophan hydroxylase [2]. 5-HT has been well investigated in a variety of organisms including vertebrates and invertebrates [3-5]. As the key molecule of the serotonergic neuroendocrine system, 5-HT not only works as a neurotransmitter, but also modulates various biological processes such as mood [6], energy metabolism [7], memory, immunity [8] and behaviors [9] in vertebrates.

The serotonergic neuroendocrine system has been reported to be indispensable for the regulation of stress response in vertebrates and invertebrates [10-12]. When exposed to a stressor, serotonergic neuroendocrine system will be activated and then release 5-HT to modulate the response. The released 5-HT binds a variety of 5-HT

receptors, and then triggers the downstream pathway to modulate physiological and metabolic adaptation to adverse conditions [13]. For example, lipopolysaccharide (LPS) stimulation would promote the release of 5-HT from monocytes of human, and enhance the capacity of cells to produce IL-6 and TNF- α [14]. In invertebrates, 5-HT has been reported to be involved in the response against various stresses (starvation, infection and heat) in arthropod and nematode [11, 12, 15, 16]. For instance, the level of 5-HT is enhanced in cell supernatants after the insect haemocytes exposed to LPS, and the elevated 5-HT modulates insect haemocyte phagocytosis via two different 5-HT receptors including 1B and 2B [1]. High-temperature treatment could induce the release of neuronal 5-HT, which would activate HSF1 and initiate the heat shock response in *caenorhabditis elegans* [17]. Apparently, 5-HT is well known for its crucial role in the regulation of stress response in both vertebrates and invertebrates. However, little is known about the modulation of 5-HT in marine molluscs, especially under environmental stress such as air exposure.

Marine molluscs living in the intertidal zones must face the harsh and dynamically environments, which can be influenced by a variety of physical factors such as salinity, pH, dissolved oxygen and temperature [18, 19]. Among all these environmental factors, air exposure is considered as one of the most deathful threats for molluscs [20]. During air exposure, molluscs have to experience a serious of stressors including increased solar radiation, higher temperature, starvation, loss of water, and hypoxia, which can cause damage to themselves [21-24]. In order to adapt to air exposure, neuroendocrine system has been proved to be responsible for the regulation of

responses to this stress [25, 26]. For example, the catecholaminergic neuroendocrine system would release catecholamines to cope with air exposure in scallop *Chlamys farreri*. Noradrenaline and dopamine concentrations in the haemolymph of scallop *C. farreri* increased significantly after air exposure [25]. However, the roles of serotonergic neuroendocrine system against air exposure in marine molluscs are still not clear.

Pacific oyster *Crassostrea gigas*, living in the intertidal zones, is distributed all over the world, which is one of the most important marine molluscs with the highest production [27]. The special habitat and wide distribution indicate the successful adaptation of oyster to the environment, making it an ideal experimental model for the investigation of the stress response of marine molluscs in estuarine regions [18, 19]. Because several physiological processes regulated by neuroendocrine system have been proved to be responsible for environmental stress [28], the investigation of the modulation of serotonergic neuroendocrine system in oyster will be conducive to know the underlying mechanism of neuroendocrine system against air exposure in *C. gigas*. The main purposes of this study are to (1) identify the neurotransmitter 5-HT in *C. gigas*, (2) determine the fluctuation of 5-HT and its response to air exposure in *C. gigas*, and (3) understand the modulation role of 5-HT against air exposure stress.

2. Materials and methods

2.1 Oysters

Pacific oysters *C. gigas* with an average shell length of 15 cm were collected from a local aquatic farm in Qingdao, Shandong province, China. They were kept in aerated

seawater (about 20°C) for 2 weeks before processing.

2.2 Air exposure and 5-HT stimulation

In air exposure stimulation experiment, one hundred and thirty-five oysters were exposed to air with 40-50% relative humidity (r. h.) at 20°C. Nine oysters were randomly sampled and divided into three parallels at 0 h, 3 h, 6 h, 12 h, 24 h, 2 d, 3 d, 4 d, 5 d and 6 d post air exposure, respectively. The haemolymph was collected from each parallel and centrifuged at 1000 g, 4°C for 10 min to collect haemocytes and supernatant. The haemocytes were kept on ice for apoptosis assay, and the supernatants were stored at -80°C for subsequent measurements of 5-HT concentration, SOD activity and H₂O₂ level.

In 5-HT treatment experiment, one hundred and twenty oysters were divided into two groups. Sixty individuals receiving an injection of 100 µL of 5-HT (10⁻⁵ mol/L in filtered seawater, Sigma Aldrich, USA) were designed as 5-HT treatment group, while the rest of sixty individuals received an injection of 100 µL of filter sterilized (0.22 µm pore size) seawater (FSSW) and were employed as control group. These oysters were returned to seawater, and nine oysters were randomly sampled and divided into three parallels at 3 h, 6 h, 12 h, 24 h and 48 h, respectively. Six individuals sampled at 0 h were used as blank group.

For the 5-HT treatment in air exposure experiment, another one hundred and eighty oysters were equally divided into three groups. After air exposure for two days, sixty individuals received an injection of 100 µL of 5-HT (10⁻⁵ mol/L) and they were regarded as 5-HT group, another sixty individuals receiving an injection of the same volume of FSSW were set as control group. The rest sixty oysters were employed as blank group. Nine oysters in each group were randomly sampled and divided into three parallels at 0 h, 3 h, 6 h, 12 h, 24 h and 48 h, respectively. The haemolymph was

collected from each parallel and centrifuged at 1000 *g*, 4°C for 10 min to harvest haemocytes and supernatant. The haemocytes were used for apoptosis assay, and the supernatant was stored at -80°C for enzyme SOD activity and H₂O₂ level measurements.

2.3 Quantification of 5-HT concentration in haemolymph of *C. gigas*

The concentration of 5-HT in haemolymph of *C. gigas* was measured following the instruction of serotonin ELISA kit (KA1894, Abnova, Taiwan). Briefly, 25 µL of acylated standards or haemolymph sample, 500 µL of acylation buffer and 25 µL of acylation reagent were pipetted into the reaction tubes, mixed thoroughly and incubated at room temperature for 15 min. The aliquots of the mixtures were added into the wells of the serotonin microtiter strips, and mixed thoroughly with 100 µL of the serotonin antiserum. After incubated at room temperature for 30 min, the contents in the wells were discarded and the wells were washed three times with washing buffer. At last, 100 µL of the chromogenic substrate (containing tetramethylbenzidine, substrate buffer and hydrogen peroxide) was added into all wells and incubated for 15 min, and then 100 µL of the stop solution H₂SO₄ (0.25 mol/L) was added to ensure a homogeneous distribution of mixtures. The absorbance of the mixtures in the wells was measured at 450 nm, and the quantification of 5-HT in haemolymph was conducted by comparing their absorbance with a reference curve prepared with the known standard concentrations.

2.4 Immunohistochemistry (IHC) localization of 5-HT in different tissues

Seven tissues including hepatopancreas, gonad, mantle, labial palp, adductor muscle, gill and visceral ganglion from nine untreated healthy oysters were collected for the IHC assay of 5-HT. Meanwhile, mantle from six individuals was also collected on the 1st d and 4th d post air exposure, respectively. These tissues were fixed in

Bouin's solution (picric acid saturated solution: formaldehyde: glacial acetic acid=15:5:1) and decolored in 70% Ethanol 6 times with 2 h each time. The samples were rehydrated in successive ethanol (80%, 95% and 100%) and soaked in xylene twice before embedded in paraffin.

The paraffin-samples were sliced in 5 μm and dried at 37°C overnight. The sections were deparaffinized in xylol and xylol: ethanol (1:1), following by rehydration in a successive dilution of 95%, 80%, 70%, 50%, 30% ethanol and distilled water. After incubation at 58°C for an hour, the sections were immersed in 0.01 mol/L PBST (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4 , 2 mmol/L KH_2PO_4 with 0.1% Tween 20, pH 7.6) for 5 minutes, and dipped in 0.01 mmol/L citrate buffer ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ and $\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$) at 120°C for 6 min for antigen renaturation.

After wash with PBST for three times, the sections were blocked with 3% bovine serum albumin (Aikbbio, China) at room temperature for 30 min, and then incubated with goat anti-serotonin antibody (ImmunoStar, USA, diluted 1:1000 with 3% BSA in PBS) at 37°C for 60 min. After rinse by PBST for three times, each section was incubated with Alexa Fluor 488-labeled donkey-anti-goat antibody (Life Technology, USA, diluted 1:1000 in 3% BSA in PBS) at 37°C for 50 min. The slices were washed with PBST for three times, blocked with 50% glycerin and finally observed with an Olympus BX51 fluorescence microscope (Olympus, Japan).

2.5 Haemocyte apoptosis assay by flow cytometry

The apoptotic index of haemocyte was determined according to the instructions of Annexin V-Alexa Fluor/PI Apoptosis Detection Kit (CWBIO, China) [29]. Briefly, the haemocytes were resuspended in modified-L15 medium (media L15 with 20.2 g/L NaCl, 0.54 g/L KCl, 0.6 g/L CaCl_2 , 3.9 g/L MgCl_2 , 1.0 g/L MgSO_4) and adjusted to a final concentration of 1×10^6 cells/mL. An aliquot of 100 μL suspension was stained

with 3 μL of Annexin V-Alexa Fluor 488 and 10 μL Propidium Iodide (PI) in darkness at room temperature for 15 min. The apoptotic index was analyzed using a flow cytometer (BD FCM, USA).

2.6 Detection of SOD activity and H_2O_2 level in haemolymph supernatant

The activity of superoxide dismutase (SOD) was measured by the T-SOD Assay Kit (Jiancheng, Nanjing, China). One SOD activity unit was defined as the enzyme amount causing 50% inhibition in 1 mL reaction solution, and was expressed as unit activity per mL of serum in the sample (U/mL) [30]. The hydrogen peroxide (H_2O_2) in haemolymph was detected by Hydrogen Peroxide Assay Kit (Beyotime, China) according to the operation instruction [31]. The H_2O_2 level assay was based on formation of a violet complex between xylenol orange and ferric iron, which was produced by the hydrogen peroxide.

2.7 Survival rate assay

The survival rate of oysters under air exposure was counted post the injection of 5-HT. Briefly, three hundred individuals were randomly divided into three groups. In the 5-HT group, one hundred oysters received an injection with 100 μL of 5-HT (10^{-5} mol/L in filtered seawater) in the adductor muscles every other day (1 d, 3 d and 5 d) during air exposure. In control group, one hundred oysters received an injection with 100 μL of FSSW every other day (1 d, 3 d and 5 d) during air exposure. The remaining one hundred oysters were employed as blank group. The mortality of oysters (the dead oyster failed to remain closed when prodded with a pipette) was conducted every day.

2.8 Statistical analysis

Data were expressed as mean S.D. ($N = 3$) and tested by one-way analysis of variance (one-way ANOVA). Letters (a/b/c, etc.) were marked to illustrate the

statistical significance ($p < 0.05$).

3. Results

3.1 The change of 5-HT concentration in oyster haemolymph during air exposure

The concentration of 5-HT in haemolymph of oyster during air exposure was evaluated (Fig. 1). It was about 76.98 ± 12.20 nmol/L in the haemolymph of oysters without any treatment, while increased significantly at 6 h (119.08 ± 16.86 nmol/L, $p < 0.05$) and 24 h (121.10 ± 13.97 nmol/L, $p < 0.05$) after air exposure. The 5-HT concentration decreased at 2nd d, and began to rise again and reached the highest level at 3rd d (136.09 ± 21.08 nmol/L, $p < 0.05$). At 5th d (133.86 ± 17.86 nmol/L, $p < 0.05$) to 6th d (121.51 ± 38.59 nmol/L, $p < 0.05$) after air exposure, the 5-HT concentration in haemolymph kept in a higher level in comparison with that in control group.

3.2 The localization of 5-HT in tissues and mantle of oysters after air exposure

The localization of 5-HT in tissues was detected by immunohistochemistry assay. The positive signals (green) of 5-HT were mainly distributed in the middle region of visceral ganglion, the edge of mantle, the gaps among hepatic tubules of hepatopancreas, but randomly and weakly in the gill, as well as the exterior structure of labial palps and gonad (Fig. 2). No positive signal was detected in adductor muscle. The change of 5-HT in the mantle of oysters after air exposure was also detected by immunohistochemistry assay (Fig. 3). The positive signal of 5-HT was initially appeared in the edge of mantle at 0 d, 1st d post air exposure, and it increased significantly along with the duration of exposure and could be observed in the middle of mantle at 4th d post air exposure.

3.3 The change of haemocyte apoptotic rate after 5-HT and air exposure treatments

The apoptotic rate of oyster haemocytes was measured after 5-HT and air exposure treatments. In the 5-HT injection experiment, the apoptotic rate was $21.37 \pm 0.38\%$ ($p < 0.05$), $18.26 \pm 0.32\%$ ($p < 0.05$), $15.97 \pm 0.57\%$ ($p < 0.05$), $18.10 \pm 0.35\%$ ($p < 0.05$) at 6 h, 12 h, 24 h and 48 h post injection, respectively, which was 0.90-fold, 0.93-fold, 0.67-fold and 0.95-fold of that in the control group. The apoptotic rate of haemocytes was significantly decreased at 6-48 h after 5-HT injection (Fig. 4A).

The changes of apoptosis rate of oyster haemocytes were measured after injection of 5-HT during air exposure from 3st d to 5th d (Fig. 4B). After air exposure, the apoptotic rate began to increase significantly at 6 h ($28.10 \pm 0.26\%$, $p < 0.05$), fell back to $20.17 \pm 0.15\%$ ($p < 0.05$) at 24 h, and then enhanced significantly at 48 h ($27.27 \pm 0.47\%$, $p < 0.05$). After the treat with 5-HT during air exposure, the apoptotic rate of oyster haemocytes was declined significantly at 6 h ($19.90 \pm 0.56\%$, $p < 0.05$) compared to that in the air exposure group, reached the lowest level at 24 h ($16.23 \pm 0.38\%$, $p < 0.05$), and then increased but still significantly lower than those in the other two groups at 48 h ($21.33 \pm 0.12\%$, $p < 0.05$).

3.4 The changes of SOD activity and H₂O₂ level in haemolymph after 5-HT stimulation or air exposure

After the injection of 10^{-5} mol/L 5-HT, the SOD activity and H₂O₂ level in haemolymph of oyster changed significantly. The activity of SOD in haemolymph of oyster was 114.21 ± 3.13 U/mL ($p < 0.05$), 126.55 ± 5.70 U/mL ($p < 0.05$) and 127.04 ± 7.02 U/mL ($p < 0.05$) at 3 h, 6 h, and 24 h post injection, respectively, which was 0.92-fold, 1.43-fold and 1.53-fold higher than that in the control group (Fig. 5A). The level of H₂O₂ was declined significantly ($p < 0.05$) in the 5-HT group at 6 h (2.02 ± 1.14 μ mol/L), 12 h (2.40 ± 0.69 μ mol/L), 24 h (2.00 ± 1.11 μ mol/L) and 48 h (1.86 ± 0.91 μ mol/L) post injection in contrast to that in the blank group, whereas it did not

change significantly in comparison with control group (Fig. 5D).

The activity of SOD and level of H₂O₂ in haemolymph were measured after air exposure, and both of them increased after air exposure. SOD activity increased immediately and reached the peak (128.42 ± 1.98 U/mL, $p < 0.05$) at 1st d after air exposure, which was 1.55-fold of that at 0 d (Fig. 5B). The level of H₂O₂ elevated significantly and reached the first peak at 1st d (16.01 ± 1.16 μ mol/L, $p < 0.05$), and then fell back to 9.27 ± 1.07 μ mol/L ($p < 0.05$) at 3rd d after air exposure, which was almost the normal concentration. SOD activity and H₂O₂ level increased significantly again in the last three days, and reached the second peak (123.89 ± 3.74 U/mL, 27.39 ± 2.89 μ mol/L, $p < 0.05$) at 5th d, respectively (Fig. 5B and Fig. 5E).

In order to explore whether 5-HT could regulate the redox reaction in oyster after air exposure, SOD activity and H₂O₂ level in haemolymph were determined at 3-5th d after the oysters were treated by air exposure and 5-HT. The activity of SOD began to increase at 3 h (133.61 ± 1.88 U/mL, $p < 0.05$), and reached the highest level at 24 h (149.75 ± 1.33 U/mL, $p < 0.05$) after oysters were treated with 5-HT which was 1.43-fold of that in the control group (Fig. 5C). It was restored to the original level of the control group at 48 h. The level of H₂O₂ in 5-HT group was decreased significantly at 6 h (10.21 ± 1.53 μ mol/L, $p < 0.05$) after air exposure, and then kept at a lower level at 12 h (7.13 ± 0.58 μ mol/L, $p < 0.05$) and 48 h (14.39 ± 2.22 μ mol/L, $p < 0.05$) in comparison with that in the control group (Fig. 5F).

3.5 The survival rate of oyster after the stimulation of 5-HT during air exposure

The survival rate of oysters under air exposure was counted after stimulation of 5-HT. In the first two days after injection, there were only a few oysters died, and almost 98% of oysters survived well in all the groups (Fig. 6). At 3rd d, the survival rate of oysters in 5-HT group and FSSW group was 97% and 96%, respectively,

which was higher than that in the air exposure group (85%). The mass mortality of oysters was noticed in air exposure and FSSW group at 4-6th d, respectively. While the survival rate of oysters was still 91%, 82% and 50% in 5-HT group at 4-6th d, which was higher than that in air exposure group (71% at 4th d, 63% at 5th d and 36% at 6th d) and control group (80% at 4th d, 72% at 5th d and 36% at 6th d), respectively.

4. Discussion

Pacific oysters *C. gigas* live in the intertidal environment with an experience period of exposure to air [32]. The continuous air exposure induces hypoxia–reduction of dissolved oxygen and water loss [22], and oysters have to experience a variety of biological changes in response to the stress, in which neuroendocrine system plays important roles in the regulation [25]. 5-HT, a neurotransmitter in serotonergic neuroendocrine system, is an important modulator in many processes including stress adaption [33], energy metabolism [7], immunity [8] and behaviors [9] in vertebrates and invertebrates [15, 34]. Accumulated evidences have demonstrated that 5-HT plays a major role in the regulation of responses to external and internal adverse stress. In order to cope with environment stress, 5-HT would modulate a series of physiological processes including metabolic processes, behaviors and immune responses [35-37]. However, whether 5-HT exists in marine molluscs and its relation to the resistance to stress such as air exposure remain unclear and need urgent study.

Available evidences have demonstrated that 5-HT is widely distributed in both animal and plant kingdoms [38-41]. In human, 5-HT is mainly located in the enterochromaffin cells of the gastrointestinal tract, the central nervous system and platelets [39]. It has been reported that 5-HT is localized in the ganglionic tissues of mollusk *Buccinum undatum* and *Helix pomatia* [42]. In the present study, 5-HT was

found to be widely distributed in oyster tissues including haemolymph, mantle, gill, labial palps, gonad, hepatopancreas and visceral ganglion. The wide expression of 5-HT in various tissues possibly provided evidences for its importance in the modulation of oysters. Interestingly, the content of 5-HT in the haemolymph of oysters (76.98 ± 12.20 nmol/L) was significant higher than that in other species. For instance, the 5-HT level in rat serum was only 47 nmol/L [43]. These results further suggested that 5-HT might play a vital role in the maintenance of homeostasis and adaption of stress in oysters.

It has been reported that environmental stress could activate serotonergic neuroendocrine system in vertebrates and invertebrates [8, 44, 45]. In mammals, the extracellular level of 5-HT in brain usually increases after exposure to psychosocial stresses. In the present immunohistochemistry assay, 5-HT was initially expressed in the edge of the mantle at 1st d post air exposure, and significantly increased along with the duration of exposure, which could be observed in the middle of the mantle. 5-HT concentration in haemolymph of oysters was also increased significantly at 6 h and 24 h post air exposure. And the 5-HT concentration in haemolymph was significantly increased from 76.98 to 135.04 nmol/L during air exposure. There have been some evidences about the increase of 5-HT at the early stage of stress response in mollusc or other species [8, 46]. The significant increase of 5-HT concentration was observed inside the cells of *Tetrahymena* at 1 h after exposure to heat stress [47]. All these phenomena showed that oysters were in a stress state at the early stage of air exposure, and the changes of 5-HT concentration and distribution in mantle or haemolymph would enable the oysters to maintain homeostasis. In mollusc, the changes of physiological contexts have been observed in haemolymph and mantle to response against the air exposure [38, 48, 49]. For instance, air exposure induced a significant

increase of H₂O₂ production in haemolymph and decrease of phagocytic index and haemocyte density in European abalone *Haliotis tuberculata* [49]. Moreover, the calcium concentration increased from 10 to 100 mmol/L [48] and the oxygen tension of water declined rapidly in the mantle cavity during air exposure of intertidal bivalves [32]. The physiological and biochemical alterations of haemolymph and mantle in oysters suggested that these tissues were important for oysters to response against air exposure. The significant increasing level of 5-HT in haemolymph and mantle of oyster suggested that it might play important role in response to air exposure. It was reported that the turnover of 5-HT in the ADF and ASG sensory neurons of *C. elegans* increased under hypoxic conditions or exposure to pathogenic bacteria through the utilization of the receptor 7 of 5-HT that mediated hypoxia response [15]. Twelve receptors of 5-HT have been annotated in the oyster genome [18], and five of them have been identified in our lab (data not shown). All these results suggested that air exposure could activate serotonergic neuroendocrine system in oysters, and triggered the release of 5-HT to response against air exposure.

It has been reported that 5-HT could regulate many physical processes including phagocytosis and apoptosis in vertebrates and invertebrates [1, 50]. For instance, 5-HT could prevent apoptosis in human monocytes against chronic inflammation in atopic dermatitis [51]. The addition of 5-HT to the seawater could activate serotonergic neurons in the apical ganglion of gastropod *Ilyanassa obsoleta*. 5-HT could mediate the activity of endogenous nitric oxide synthase to generated nitric oxide (NO), and then the synthesized NO could modulate apoptotic signaling pathways [52]. In the present study, the exogenous 5-HT was also found to be able to inhibit the apoptosis of oyster haemocytes in normal condition. Moreover, continuous air exposure was found to induce apoptotic effects on haemocytes. Accumulated

reports have shown that environmental stress triggered the apoptosis [53, 54]. For instance, hypoxic exposure increased apoptosis rate in the rat heart and human adenocarcinoma HT29 cells [55, 56]. Apoptosis is involved in the maintenance of cellular homeostasis and responds to internal and external stimuli. The increasing of apoptosis rate would cause several harmful effects including diseases and pathologies [53]. Therefore the inhibition of air exposure-induced apoptosis effect is crucial for survival of oysters. In the present study, the injection of 5-HT could inhibit the apoptosis of haemocytes at 24 h after air exposure. These results collectively indicated that the high concentration of 5-HT could negatively modulate the apoptosis of oyster haemocytes at the late stage of the response against air exposure.

Except for the induction of apoptosis, 5-HT exhibits powerful antioxidant activity which could protect vertebrates from oxidative stress [33, 57]. It has been reported that the antioxidant enzymes, glutathione peroxidase (GPx) and SOD, was reduced in the 5-HT deficient mice [43]. 5-HT could increase SOD production in vascular smooth muscle via an NADPH oxidase pathway, which leads to an increase of extracellular SOD levels [58]. In the present study, the activity of SOD was increased in the serum of oysters after injection of 5-HT. Moreover, continuous air exposure increased the activity of SOD in haemolymph of oysters. Accumulating evidences demonstrated that air exposure altered the oxidant–antioxidant status and triggered antioxidant responses of related enzymes. An increase of antioxidant enzyme-SOD activity was observed in the tissues of muscle, hepatopancreas and haemolymph during air exposure in the subantarctic stone crab *Paralomis granulosa* [59]. In the present study, 5-HT could upregulate the activity of SOD in haemolymph of oysters after air exposure. These results suggested that 5-HT played a role in oysters by controlling the release of enzyme SOD in haemolymph after air exposure to maintain

tissue redox. In the present study, 5-HT didn't affect the level of H_2O_2 in serum of oysters in normal condition, but it could alleviate the toxicity of H_2O_2 in serum of oysters after air exposure. H_2O_2 is a metabolic by-product of ROS that serves as a key regulator for a number of oxidative stress-related states [60]. 5-HT has been reported to inhibit the production of H_2O_2 and reactive oxygen from neutrophils [61]. And 5-HT and its receptor could stimulate the generation of reactive oxygen species (ROS) including H_2O_2 and superoxide anions, and then regulate the chronic hypoxia-induced pulmonary hypertension [62]. The present results demonstrated that 5-HT could enhance the resistance to oxidative stress by decreasing the accumulation of H_2O_2 in oysters after exposure to air. As a whole, 5-HT could resist oxidative stress under air exposure through increasing the activity of SOD and reducing accumulation of H_2O_2 in haemolymph of oysters.

The protective effect of 5-HT was tested by surveying the survival rate of oysters after the stimulation of 5-HT under air exposure. The survival rate of oysters decreased dramatically at 3rd d in air exposure group and 4th d in FSSW group respectively. But the survival rate in 5-HT group began to reduce significantly at 5th d, and it was higher than that in the other two groups at 6th day. It was evident that the stimulation of 5-HT could increase the survival of oysters upon exposure to air stress. In human, 5-HT could rescue monocytes from apoptosis of patients with chronic atopic dermatitis, and thus extended their life-span [51]. In addition, 5-HT could reduce mortality in endotoxic shock of neutrophils [63]. It has also been reported that the optogenetic stimulation of serotonergic neurons in *C. elegans* could increase its survival upon subsequent exposure to a lethal dose of heat stress [17]. The present results clearly confirmed that the increasing 5-HT level could provide protection for oysters against air exposure in a certain period of time.

In conclusion, the level of 5-HT in mantle and haemolymph of oyster *C. gigas* was significantly increased in response to air exposure. Exogenous 5-HT could reduce the apoptotic effect of haemocytes and accumulation of H₂O₂ in haemolymph, and improve the activity of SOD after air exposure. Moreover, the increasing 5-HT level could reduce the mortality of oysters during air exposure, which confirmed the protective mechanisms of 5-HT. These results provided evidences to demonstrate that 5-HT could modulate the apoptotic effect and redox, and play positive modulation roles in response to air exposure in oysters.

Acknowledgements

We are grateful for all the tremendous support from laboratory members. This research was supported by the High Technology Project (863 Program, No. 2014AA093501) from the Chinese Ministry of Science and Technology, a grant (No. 31530069) from National Science Foundation of China, Dalian high level talent innovation support program (2015R020), earmarked fund (CARS-48) from Modern Agro-industry Technology Research System, and the Research Foundation for Talented Scholars in Dalian Ocean University (to L. W.).

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Figure legends

Fig.1. Temporal change of 5-HT in oyster haemolymph during air exposure. The values were indicated with mean $\pm$ SD (N = 3). The different letters (a, b, c etc.)

indicated significant difference ($p < 0.05$, ANOVA).

Fig.2. Immunofluorescence histochemistry analysis of the distribution of 5-HT in tissues: hepatopancreas, gonad, mantle, labial palp, adductor muscle, gill and visceral ganglion. The red background was stained by the Evans Blue dye which could label the cell membrane. The positive signal was shown by green fluorescence.

Fig.3. Immunofluorescence histochemistry analysis of the distribution of 5-HT in mantle under air exposure. The mantle was sampled at 0 d, 1 d and 4 d during air exposure. The red background was stained by the Evans Blue dye which could label the cell membrane. The positive signal was shown by green fluorescence.

Fig.4. Effect of *in vivo* stimulation of oysters with 5-HT and air exposure on apoptosis rate of haemocytes. A: The change of apoptosis rate in haemocytes after 5-HT stimulation. B: The change of apoptosis rate in haemocytes after 5-HT stimulation under air exposure from 3rd d to 5th d. The values were indicated with mean $\pm$ SD (N = 3) and the significant letters (a, b, c) showed significant differences ($p < 0.05$, ANOVA).

Fig.5. Effect of *in vivo* stimulation of oysters with 5-HT and/or air exposure on SOD and H₂O₂ activities in haemolymph. A: Temporal variation of SOD activity in haemolymph after injection of 5-HT. B: Temporal change of SOD activity in haemolymph after air exposure. C: Temporal variation of SOD activity in haemolymph after injection of 5-HT during air exposure from 3rd d to 5th d. D: Temporal variation of H₂O₂ level in haemolymph after injection of 5-HT. E: Temporal

change of H₂O₂ level in haemolymph after air exposure. F: Temporal variation of H₂O₂ level in haemolymph after injection of 5-HT during air exposure from 3rd d to 5th d. Vertical bars represented the mean $\pm$ SD (N=3). The different letters (a, b, c) showed significant differences ($p < 0.05$, ANOVA).

Fig.6. Survival rate of oyster after stimulation of 5-HT under air exposure for six days. None: one hundred oysters were held in air conditions; FSSW: one hundred oysters were injected 100 μ L filter sterilized (0.22 μ m pore size) sea water every other day; 5-HT: one hundred oysters were received an injection of 100 μ L 5-HT every other day.

Figure1

Figure2

visceral ganglion

hepatopancreas

mantle

gill

labial palps

gonad

Figure3

0 d

1 d

4 d

Figure4

A

B

Figure5

A

B

C

D

E

F

Figure6