Short communication

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Effect of hypoxia on hemocyte parameters of mussel (*Mytilus galloprovincialis* Lmk.) and the Pacific oyster (*Crassostrea gigas* L.) cultivated on shellfish farm (salt Lake Donuzlav, Crimea)

Kladchenko E.S., Andreyeva A.Y., Vyalova O.Y., Kukhareva T.A.

Department of Animal Physiology and Biochemistry, A.O. Kovalevsky Institute of Biology of the Southern Seas of RAS Nakhimov Avenue, 2, Sevastopol, 299011, Russia

ABSTRACT. Circulating hemocytes of the Pacific oyster (Crassostrea gigas) and mussel (Mytilus galloprovincialis) were investigated using light microscopy and flow cytometry. In both bivalve species two cell types, granular and agranular, were identified on the basis of cell distribution by size and granularity level on flow cytometric dot plots. Hypoxia (24 h) led to substantial changes in hemolymph cellular composition both in mussels and oysters. After hypoxic treatment the level of reactive oxygen species (ROS) production decreased compared to normoxia level, however DCF-DA fluorescence in agranulocytes of mussels increased. No significant changes in hemocyte mortality were observed for both species.

Keywords: hemocyte, hypoxia, oyster, mussel

1. Introduction

Hypoxia is a known issue for coastal marine systems (Stramma et al., 2010). Low dissolved oxygen negatively influences physiology of bivalve mollusks, causing spreading of diseases and increasing mortality level (Parisi et al., 2017). Stable shellfish cultivating requires regular examination of physiological state of growing mollusks. Therefore, fundamental studies investigating the effects of hypoxia on cultured bivalve species are needed.

In the present study, we characterized hemocyte morphology and physiology of cultivated bivalves (*Mytilus galloprovincialis* and *Crassostrea gigas*), using light microscopy centrifugation and flow cytometry in the context of comparative morphological and functional properties. The impact of 24 h hypoxia on hemocyte functions including mortality level and spontaneous reactive oxygen species (ROS) production were investigated.

2. Material and methods

Specimens of mussels *(M. galloprovincialis)* and the Pacific oysters *(C. gigas)* were captured on a shellfish farm (salt lake Donuzlav, Crimea). Groups of 15–20 individuals were transferred into laboratory in 50 L tanks with aerated sea water. Hypoxic conditions were conducted by bubbling of tank with mollusks with nitrogen gas reaching oxygen concentration 0.4 mg $^{L-}$1. Hypoxic conditions in the tank were maintained for 24 h.

After the end of incubation period hemolymph (0.1-1.5 ml) was withdrawn from the adductor muscle of mussels and from the heart sinus of oysters. Then samples were centrifuged at 350g for 5 min. After the final washing, drops of hemocyte pellet were placed on a glass slide and dried for 24 h on air. Slides were stained with May–Grünwald and Giemsa solutions and then examined on a light microscope (Biomed PR-2 Lum) equipped with camera (Levenhuk C NG Series). Approximately 1000 cells per slide were examined. Diameter of cells and nuclei was measured using the ImageJ 1.44 p.

For flow cytometry analysis, hemocyte concentration in sterile filtered seawater was adjusted to 1-2·10^6 cell ml^{-1}. Suspensions were analyzed by a FC500 flow cytometer (Beckman Counter). Cells were dyed with DNA-binding fluorochrome SYBR Green I (final concentration in the probe 10μM) for determination of cell types. Hemocyte mortality level was estimated using staining of cells with propidium iodide (PI). 10

*Corresponding author.
E-mail address: kladchenko_ekaterina@bk.ru (E.S. Kladchenko)

μl of 200 μg ml^{-1} PI stock solution (Sigma Aldrich) was added to 1 ml hemocyte suspension, and the sample was incubated in the dark for 40 min at 4º C before flow analysis. To test spontaneous ROS production 2-7-dichlorofluorescein-diacetate (DCF-DA) was used. 1 ml of hemocyte suspensions was incubated with 10 μl of DCF-DA solution for 30 min in the dark. The green fluorescence produced by DCF was measured on FL1 channel.

Mann–Whitney U-test was used to compare the functional and morphological characteristics of hemocytes ($p \leq 0.05$). All results are expressed as Mean ± SE.

3. Results and discussion

In mussel, two cell types, granulocytes and agranulocytes, were identified based on the existence of two subpopulations of cells differing by size and granularity level on light-scattered plots. In oyster, three types of hemocytes were described: agranulocytes, hyalinocytes and granulocytes. For both species, agranular cells were dominating cell type in hemolymph amounting 78.4 ± 8.9% in mussels and 86.7 ± 2.7 % (agranulocytes and hyalinocytes) in oysters. Agranulocytes were the smallest cell type by their diameter (8.0 ± 0.1 μm and 9.1 ± 0.1 μm in mussels and oysters respectively). They possessed round shape, large nuclei and narrow cytoplasm. Hyalinocytes of oysters were larger cells (9.7 ± 0.2 μm) with excentric nuclei and irregular shape. Granulocytes of both species contained numerous eosinophilic, basophilic and mixed-type granules and formed pseudopodia. Flow cytometry analysis demonstrated, that the agranular hemocytes of both species produce significantly less reactive oxygen species compared to granulocytes. The present work clearly confirms similarity in flow-cytometric profiles for hemocytes of marine bivalves. It is now suggested, that two major types of cells have been characterized in bivalves: granulocytes, containing granules of various size and number, and small nuclei; and agranulocytes, which do not possess granules, and have high N/C ratio (Hine, 1999).

Hypoxia caused substantial changes in hemolymph composition. After exposure to hypoxia the number of agranular cells in oyster hemolymph was 2.5 times higher compared to normoxic level. In contrast, the number of the agranular cells in hemolymph of mussels was 1.7 times lower in hypoxic probes compared to control. The level of dead cells in hemolymph did not change substantially for both species. In granulocytes of both species and in oysters` hyalinocytes, intracellular ROS concentration also decreased significantly. Probably, granulocytes and hyalinocytes are more oxygen demanding cells compared to agranulacyte. In agranulocytes of mussel ROS production was substantially higher comparing to the normoxic level. DCF-DA fluorescence in agranulocytes of oysters decreased, in contrast. Similar species differences in effect of hypoxia on ROS production were obtained by other authors (Song et al., 2010).

4. Conclusions

Thus, effect of hypoxia on hemocytes depends on the level of species tolerance to hypoxia. The results of the present work demonstrate that mussels are more tolerant to hypoxia compared to oysters.

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