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ERYTHROID CELLS IN THE HEMOLYMPH OF A BIVALVE *ANADARA KAGOSHIMENSIS* (TOKUNAGA, 1906) UNDER HYDROGEN SULFIDE LOADING: FLOW CYTOMETRY AND LIGHT MICROSCOPY

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The imbalance between organic matter oxidation and oxygen supply mediates the formation of time-stable redox zones in the water column. On the shelf, this typically occurs due to the absence of thorough vertical convection and the formation of localized decomposition zones. The functional mechanisms of the resistance of certain benthic organisms to such conditions are of particular interest. In this work, we study a bivalve *Anadara kagoshimensis* (Tokunaga, 1906) known for its tolerance to hydrogen sulfide contamination. Using flow cytometry and light microscopy, we examined the effect of hydrogen sulfide loading on morphofunctional characteristics of its erythroid cells under experimental conditions. The analysis was carried out on adult specimens with a shell height of 23–34 mm. The control group of molluscs was kept in an aquarium with an oxygen concentration of 7.0–8.2 mg $O_2 \cdot L^{-1}$ (normoxia). For the experimental group, oxygen content was first lowered to 0.1 mg $O_2 \cdot L^{-1}$ for 2 h (*via* nitrogen bubbling); then, Na_2S was added to water to a final concentration of 6 mg $S^{2-} \cdot L^{-1}$. Exposure to hydrogen sulfide revealed a significant increase in the volume of erythroid cells in *A. kagoshimensis* hemolymph (more than 40%, $p < 0.01$) accompanied by a substantial rise in fluorescence intensity of rhodamine 123 (R123) and 2′-7′-dichlorofluorescein-diacetate (DCF-DA) (2–3-fold, $p < 0.01$). This evidences for enhanced oxidative processes within cells and their possible lysis. The latter one may facilitate the release of hematin-containing granules, and hematin is capable of neutralizing sulfides. The observed response seems to be an adaptive one. A rise in values of side scatter and SYBR Green I fluorescence reflects an increase in abundance of granular inclusions within red blood cells under hydrogen sulfide loading and a gain in the functional activity of their nuclei.

Keywords: *Anadara kagoshimensis*, hydrogen sulfide, hemolymph, erythroid cells, morphology, flow cytometry

A Pacific bivalve *Anadara kagoshimensis* (Tokunaga, 1906) was first recorded off the coast of the Caucasus in 1968 [Kiseleva, 1992]. Currently, its abundant populations are primarily registered along the Caucasian and Romanian coasts of the Black Sea [Revkov, 2016]. This mollusc is actively colonizing the Sea of Azov shelf [Zhivoglyadova et al., 2021], and it has already become one of the dominant benthic forms [Revkov, 2016]. Such a rapid expansion of the Sea of Azov–Black Sea region is attributed to the species adaptive plasticity, particularly the ability to survive under low oxygen levels and hydrogen sulfide contamination.

Specialized studies have shown that *A. kagoshimensis* can maintain high energy levels in its tissues under acute hypoxia [Cortesi et al., 1992]. At the same time, the intensity of aerobic metabolism in its tissues is much lower compared to that of other bivalves [Andreenko et al., 2009]. The mollusc is resistant to hydrogen sulfide in water [Miyamoto, Iwanaga, 2017], and this is partly attributed to the presence of hematin-containing granules in its hemolymph cells [Holden et al., 1994; Vismann, 1993]. The density of these inclusions was shown to increase noticeably under experimental hydrogen sulfide loading [Soldatov et al., 2018].

Certain effects have also been revealed in the response of *A. kagoshimensis* cellular systems to hypoxia and presence of hydrogen sulfide [Soldatov et al., 2018, 2021]. Those primarily concern the functional morphology of cells: volume, specific surface area, and nuclear–cytoplasmic ratios. The present research continues earlier studies.

We aimed at assessing the effect of hydrogen sulfide on functional characteristics of erythroid cells in *Anadara kagoshimensis* hemolymph under *in vivo* experimental conditions using flow cytometry and light microscopy.

MATERIAL AND METHODS

Adult *A. kagoshimensis* specimens were studied. Bivalves were sampled in the Laspi Bay (the Crimea). Shell height ranged 23 to 34 mm (measured from the hinge to the valve edge).

Experimental design. The molluscs were divided into two groups: the control and the experimental ones. The control group was kept at an oxygen concentration of 7.0–8.2 mg $O_2 \cdot L^{-1}$. In the experimental group, oxygen content was lowered to 0.1 mg $O_2 \cdot L^{-1}$ for 2 h (*via* nitrogen bubbling); then, Na_2S was added to water to a final concentration of 6 mg $S^{2-} \cdot L^{-1}$. The presence of sulfide ions increased water alkalinity, and it was compensated by using 0.1 N HCl. Values of pH were stabilized at 8.2–8.3. Molluscs of both groups were kept under these conditions for 48 h. Water temperature was of +17…+20 °C.

Oxygen levels were monitored with a portable dissolved oxygen meter DO Meter ST300D (Ohaus, the USA). Values of pH were measured with a pocket pH-meter InoLab pH 720 (Germany). Sulfide ion concentration in water was determined potentiometrically with a sulfide-selective MSBS sensor (the Netherlands).

Hemolymph sampling. Hemolymph was sampled *via* syringe puncture of the extrapallial space. Heparin (Richter, Hungary) served as an anticoagulant. The obtained sample was divided into two parts. The first one was used to prepare smears. The second part was washed *via* centrifugation three times [500 g, 5 minutes, +4 °C, Eppendorf 5424 R refrigerated centrifuge (Germany)] to remove plasma. The cells were resuspended in sterile seawater. The obtained samples were used for cytometric studies.

Flow cytometry. A part of the erythrocyte suspension was stained with SYBR Green I (Sigma Aldrich, the USA). The final concentration in the sample was of 10 μM; the incubation in the dark lasted for 40 min. The fluorescence of this DNA dye was analyzed in the FL1 channel (excitation at 497 nm, emission at 521 nm). The pattern of distribution of cells in the suspension was classified based on their relative size: by forward scatter (FS) and side scatter (SS).

The spontaneous production of reactive oxygen species by erythrocytes was assessed by fluorescence of 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA, Sigma Aldrich). Thus, 1 mL of the erythrocyte suspension was incubated with 10 μL of DCF-DA solution for 40 min in the dark. The final dye concentration in the sample was of 10 μM. Its fluorescence was analyzed in the FL1 channel (excitation at 485 nm, emission at 525 nm).

Changes in the mitochondrial membrane potential of erythrocytes were monitored by fluorescence intensity of cells stained with rhodamine 123 (R123) (Molecular Probes, the USA). Erythrocytes were R123-stained for 40 min. The dye concentration in the sample was of 2.5 μM. Fluorescence intensity was measured in the FL1 channel (excitation at 508 nm, emission at 528 nm).

All measurements were carried out on a Cytomics FC 500 flow cytometer (Beckman Coulter, the USA) equipped with a single-phase argon laser (wavelength of 488 nm).

Light microscopy. The part of the hemolymph used to prepare smears was stained by the combined Pappenheim method (May–Grünwald + Romanowsky–Giemsa) [Zolotnitskaya, 1987]. Then, this part was used to assess the morphometric characteristics of red blood cells. Under a Biomed PR-2 Lum light microscope (magnification 100×, China–Russia) equipped with a Levenhuk C NG Series camera, large and small diameters of cells (C_1 and C_2, respectively) and their nuclei (N_1 and N_2) were measured from photographs (ImageJ 1.44p software). The sample size was 100 cells *per* smear. Based on obtained values and using established algorithms, mean cell volume (V_c) [Houchin et al., 1958], nuclear volume (V_n) [Tască, 1976], and nuclear–cytoplasmic ratio (NCR) were calculated:

$$V_c = 0.7012 \cdot \left(\frac{C_1 + C_2}{2}\right)^2 \cdot h + V_n \,,$$

$$V_n = \frac{\pi \cdot N_1 \cdot N_2^2}{6} \,,$$

$$NCR = \frac{V_n}{V_c} \,.$$

Statistical processing. Data are presented as $M \pm m$. Statistical comparisons were made using the Mann–Whitney U test. Difference was considered significant at $p < 0.05$. The data were processed and visualized in MS Office Excel 2010. Sample sizes are shown in the graphs.

RESULTS

Flow cytometry. The analysis of forward scatter (FS) and side scatter (SS) in suspensions of erythroid cells under hydrogen sulfide loading revealed an increase in both parameters (Fig. 1). In the case of SS, the differences were statistically significant ($p < 0.001$).

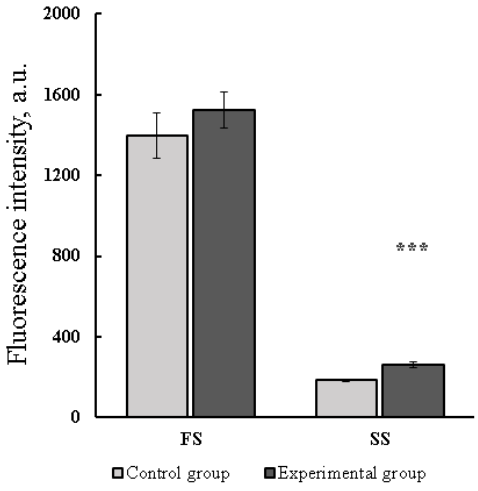

Fig. 1. Parameters of forward scatter (FS) and side scatter (SS) of suspensions of erythroid cells in *Anadara kagoshimensis* hemolymph under hydrogen sulfide loading (***, $p < 0.001$)

A noticeable rise in fluorescence intensity in the presence of H_2S was observed for SYBR Green I, R123, and DCF-DA as well (Fig. 2): 1.7-fold ($p < 0.05$), 1.9-fold ($p < 0.01$), and 3.2-fold ($p < 0.001$), respectively.

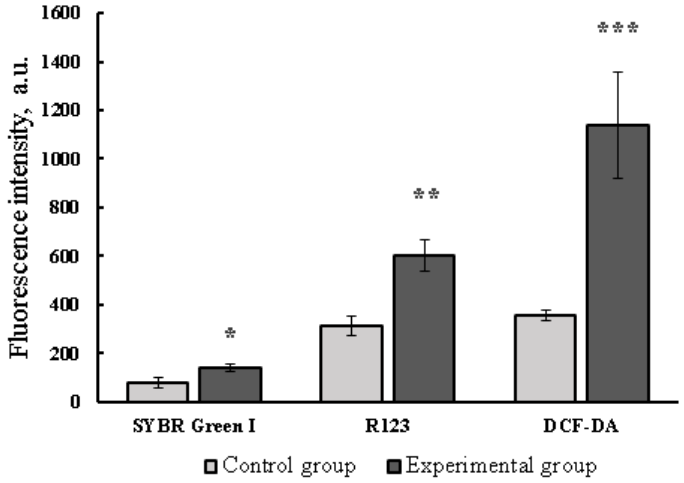

Fig. 2. Fluorescence intensity of SYBR Green I, R123, and DCF-DA suspensions of erythroid cells in *Anadara kagoshimensis* hemolymph under hydrogen sulfide loading (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

Morphometry of erythroid cells. As revealed, under hydrogen sulfide loading, there was an increase in cell volume and the number of granular inclusions in cells (Fig. 3). It was confirmed by morphometric data. Longitudinal and transverse axes of erythroid cells (C_1 and C_2) were increased by 20–21% ($p < 0.05$ for C_2) (Fig. 4). There was a boost in cell volume as well: by more than 40% ($p < 0.01$). Nuclei exhibited similar changes, but those were weakly expressed ($p > 0.05$) (Fig. 5). The calculation of NCR also revealed no statistically significant shifts: the values for the control group were of (0.120 ± 0.020), and for the experimental one, (0.090 ± 0.004).

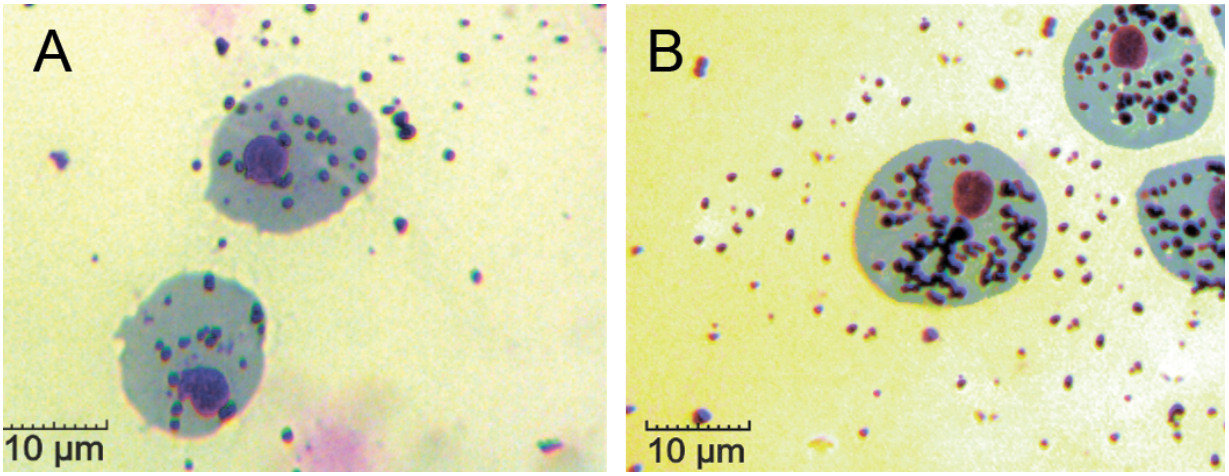

Fig. 3. Erythroid cells in *Anadara kagoshimensis* hemolymph (A, normoxia; B, hydrogen sulfide contamination)

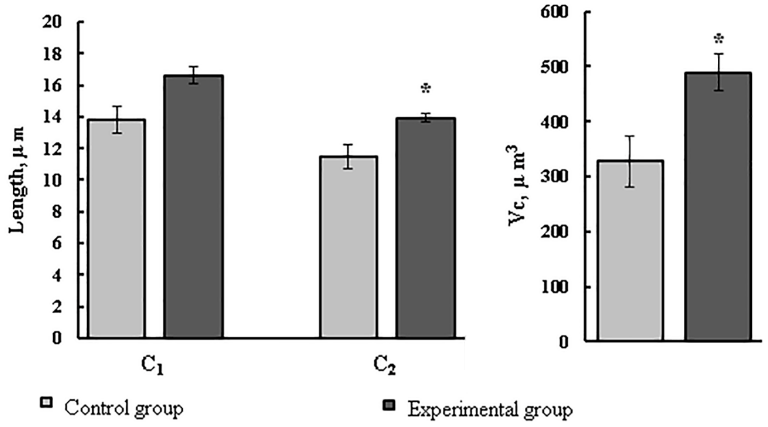

Fig. 4. Morphometric characteristics of erythroid cells in *Anadara kagoshimensis* hemolymph under normoxia and hydrogen sulfide loading (C_1, large cell diameter; C_2, small cell diameter; V_c, cell volume; *, $p < 0.01$)

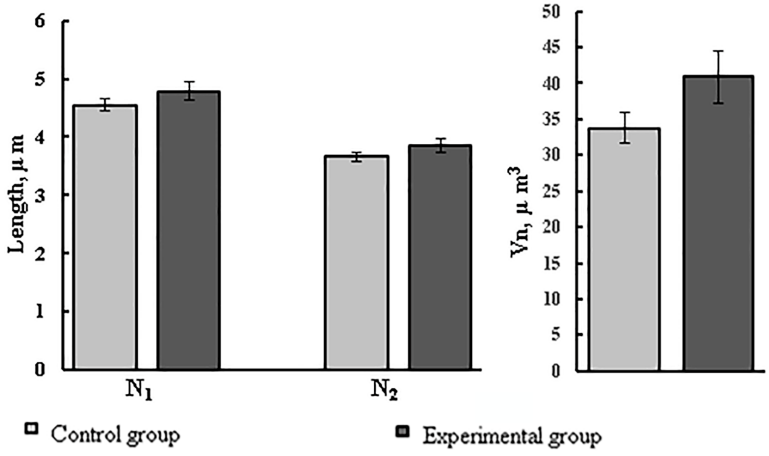

Fig. 5. Morphometric characteristics of nuclei of erythroid cells in *Anadara kagoshimensis* hemolymph under normoxia and hydrogen sulfide loading (N_1, large nucleus diameter; N_2, small nucleus diameter; V_n, nucleus volume)

DISCUSSION

The results of our experiment showed that hydrogen sulfide loading induced distinct alterations in erythroid cells of *A. kagoshimensis* hemolymph: an increase in cell volume, a rise in SS of cell suspensions, and a boost in fluorescence intensity of SYBR Green I, R123, and DCF-DA.

Typically, the presence of hydrogen sulfide in marine environment co-occurs with acute hypoxia, with the latter one known to induce erythrocyte swelling [Holk, 1996; Nikinmaa et al., 1987]. Earlier, we observed similar responses in *A. kagoshimensis* under both hypoxia alone and its combined effect with hydrogen sulfide [Soldatov et al., 2018]. In fish erythrocytes, hypoxia activates Na^+/H^+ antiporters [Salama, Nikinmaa, 1990; Val et al., 1997]. This reaction is further amplified by catecholamines (*e. g.*, adrenaline and noradrenaline), and their production can be enhanced under experimental loading. Nuclear erythrocytes were shown to contain β-adrenergic receptors through which hormones stimulate cAMP production in cells [Salama, Nikinmaa, 1990; Val et al., 1997]; typically, this results in a cell volume increase by 5–6% only [Nikinmaa et al., 1987]. Importantly, in our study, cell volume rose by more than 40%. This often precedes the apoptosis characterized by production of cell fragments called apoptotic bodies [Manskikh, 2007]. Our previous studies documented lysis of erythroid cells in *A. kagoshimensis* under hydrogen sulfide loading [Soldatov et al., 2018]. This reaction seemed to be an adaptive one, as it was accompanied by the release of granular inclusions capable of neutralizing sulfides [Holden et al., 1994; Vismann, 1993].

The observed rise in SS we recorded aligns well with the increased cytoplasmic granularity of erythroid cells, as visually confirmed earlier in hydrogen sulfide–exposed *A. kagoshimensis* [Soldatov et al., 2018]. These granules contain hematin [Vismann, 1993] which reacts with sulfides to form sulfur:

$$2Fe^{3+} + 3S^{2-} \rightarrow Fe_2S_3 \,,$$
$$2Fe_2S_3 + 3O_2 = Fe_2O_3 + 6S^0 \,.$$

Given that sulfur accumulation has been recorded in other marine invertebrates exposed to sulfides [Powell et al., 1980], similar reactions may occur in *A. kagoshimensis* hemolymph.

The increase in SYBR Green I fluorescence intensity in *A. kagoshimensis* erythrocytes in the presence of hydrogen sulfide we registered is currently difficult to interpret, since the mechanism of interaction between this fluorochrome and DNA molecule is not fully known. However, several authors suggest that in most cases, this phenomenon reflects enhanced functional activity of the cell nucleus [Cerca et al., 2011]. Given that the volume of nuclei in *A. kagoshimensis* erythroid cells increased under H_2S loading alongside with cytoplasmic granularity of the cell, we may reasonably accept this perspective as a working hypothesis.

A significant rise in R123 and DCF-DA fluorescence in erythroid cells of the mollusc's hemolymph under hydrogen sulfide loading we observed indicates an increase in mitochondrial membrane potential and levels of reactive oxygen species. Overall, this evidences for a substantial intensification of oxidative processes within cells which would likely lead to their lysis. The previously noted excessive increase in cell volume seems to reflect this process. The destruction of these cells is accompanied by the release of hematin-containing granular inclusions, and hematin is capable of neutralizing sulfides in *A. kagoshimensis* hemolymph. This response appears to be an adaptive mechanism specific to red blood cells. In other somatic tissues (gills, hepatopancreas, and foot muscle), it is virtually absent [Soldatov et al., 2022].

Conclusion. Experimental findings revealed as follows: under hydrogen sulfide loading, the volume of erythroid cells in *Anadara kagoshimensis* hemolymph significantly increased and was accompanied by a substantial rise in R123 and DCF-DA fluorescence. This indicates enhanced oxidative processes in cells and their possible lysis. The latter one seems to facilitate the release of hematin-containing granular inclusions, and hematin is capable of neutralizing sulfides. This observed response appears to have an adaptive significance. An increase in side scatter and SYBR Green I fluorescence evidences for a higher number of granular inclusions in erythroid cells in the presence of hydrogen sulfide, as well as elevated functional activity of their nuclei.

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ЭРИТРОИДНЫЕ ЭЛЕМЕНТЫ ГЕМОЛИМФЫ ДВУСТВОРЧАТОГО МОЛЛЮСКА *ANADARA KAGOSHIMENSIS* (TOKUNAGA, 1906) В УСЛОВИЯХ СЕРОВОДОРОДНОЙ НАГРУЗКИ: ПРОТОЧНАЯ ЦИТОМЕТРИЯ И СВЕТОВАЯ МИКРОСКОПИЯ

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Нарушение баланса между окислением органического вещества и поступлением кислорода приводит к формированию в водной толще устойчивых во времени редокс-зон. На шельфе это обычно происходит вследствие отсутствия сквозной вертикальной конвекции и образования локальных зон гниения мёртвого органического вещества. Функциональные аспекты устойчивости ряда бентосных организмов к подобным условиям представляют определённый интерес. В настоящей работе исследован двустворчатый моллюск *Anadara kagoshimensis* (Tokunaga, 1906), способный переносить условия сероводородного заражения. При помощи методов проточной цитометрии и световой микроскопии экспериментально изучено влияние сероводородной нагрузки на морфофункциональные характеристики эритроидных элементов моллюска. Работа выполнена на взрослых особях с высотой раковины 23–34 мм. Контрольную группу *A. kagoshimensis* содержали в аквариуме с концентрацией кислорода 7,0–8,2 мг $O_2 \cdot л^{-1}$ (нормоксия). У опытной группы сначала в течение 2 ч понижали уровень кислорода до 0,1 мг $O_2 \cdot л^{-1}$ (барботаж воды азотом). Затем в воду вносили Na_2S до финальной концентрации 6 мг $S^{2-} \cdot л^{-1}$. При наличии сероводорода выявлен значительный рост объёма эритроидных элементов гемолимфы анадары (более 40 %, $p < 0{,}01$), происходящий на фоне существенного повышения величины флуоресценции родамина 123 (R123) и 2′-7′-дихлорфлуоресцеин-диацетата (DCF-DA) (2–3 раза, $p < 0{,}01$), что отражает усиление окислительных процессов в клетках и их возможный лизис. Последний позволяет освобождать гранулы, содержащие гематин, который способен нейтрализовать сульфиды. Отмеченная реакция, по-видимому, имеет адаптивное значение. Рост величин бокового светорассеяния и флуоресценции SYBR Green I отражает увеличение числа гранулярных включений в клетках красной крови при наличии сероводорода и повышение функциональной активности их ядер.

Ключевые слова: *Anadara kagoshimensis*, сероводород, гемолимфа, эритроидные элементы, морфология, проточная цитометрия