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MORPHOMETRIC CHARACTERISTICS OF ERYTHROID ELEMENTS OF *ANADARA KAGOSHIMENSIS* (TOKUNAGA, 1906) HEMOLYMPH UNDER CONDITIONS OF HYDROGEN SULFIDE LOADING

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The effect of hydrogen sulfide loading on the morphometric characteristics of erythroid elements of *Anadara kagoshimensis* (Tokunaga, 1906) hemolymph was studied experimentally. The work was carried out on adult molluscs with a shell height of 26–38 mm. Molluscs of the control group were kept in an aquarium with oxygen concentration of 7.0–7.1 mg $O_2 \cdot L^{-1}$ (normoxia). Molluscs of the experimental group were exposed to hydrogen sulfide loading created by Na_2S donor dissolving in water to a final concentration of 6 mg $S^{2-} \cdot L^{-1}$. A day later, the oxygen level in water amounted to 1.8 mg $O_2 \cdot L^{-1}$, and hydrogen sulfide was not detected. Some of molluscs were subjected to repeated hydrogen sulfide loading by Na_2S adding up to a final concentration of 9 mg $S^{2-} \cdot L^{-1}$. By the end of the second day, 1.9 mg $S^{2-} \cdot L^{-1}$ and 0.03 mg $O_2 \cdot L^{-1}$ (trace oxygen concentration) were recorded in water. Under conditions of short-term hydrogen sulfide loading (the first day), the population of *A. kagoshimensis* erythroid elements became more heterogeneous. In the hemolymph, the content of micro- and macrocytes increased; the number of cells with an altered shape and low content of granular inclusions in the cytoplasm rose. The number of free hematin granules in the hemolymph significantly increased. The mean cell volume (V_c) rose by more than 20%. Exposure to increased concentration of sulfides for two days led to a noticeable decrease in V_c, which is determined by a significant reduction in the population of macrocytes in the hemolymph of molluscs.

Keywords: molluscs, *Anadara kagoshimensis*, hydrogen sulfide, hemolymph, erythroid elements

The occurrence of an extensive redox zone (a chemocline zone) in the Black Sea fundamentally distinguishes this water area from other ones in the World Ocean. It is characterized by a combination of conditions of acute hypoxia with hydrogen sulfide contamination [Podymov, 2005]. The chemocline zone is usually located at depths of 100–150 m. A similar set of conditions can be formed in the shelf zone [Zaika et al., 2011]. Most often, it is a consequence of the lack of end-to-end vertical convection and the formation of local areas of decay of dead organic matter [Orekhova, Konovalov, 2018]. Upwelling processes contributing to the accidental transport of hydrogen sulfide-contaminated deepwater into the coastal zone should also be taken into account [Orekhova, Konovalov, 2018].

Of particular interest are organisms capable of living in conditions of hydrogen sulfide contamination and extremely low oxygen concentrations. In this regard, an invasive bivalve *Anadara kagoshimensis* (Tokunaga, 1906) stands out. First found in the Black Sea in 1968 [Kiseleva, 1992], it has become

one of the leading forms of benthos by now [Revkov, 2016]. Under experimental conditions, *A. kagoshimensis* showed high resistance not only to acute forms of hypoxia [Cortesi et al., 1992; Isani et al., 1989], but also to hydrogen sulfide loading [Miyamoto, Iwanaga, 2017; Nakano et al., 2017]. These are supposed to be the reasons for its wide distribution in the problematic water areas of the Black Sea and Sea of Azov [Revkov, 2016].

The tolerance of molluscs to acute forms of hypoxia and anoxia is well studied. It is shown to be based on the ability of their body to couple the processes of protein and carbohydrate metabolism. This is evidenced by an increase in NH_4^+ production [Chew et al., 2005], a rise in the activity of alanine and aspartate aminotransferase [Soldatov et al., 2009], a gain in processes of transamination of glutamate and alanine [Hochachka, Somero, 2002], and a formation of alanine and succinate as end-products [Buck, 2000].

The ability of molluscs to compensate for the occurrence of hydrogen sulfide in water is not fully investigated. As shown, their hemolymph contains a special protein and hemoglobins which are insensitive to hydrogen sulfide [Arp, Childress, 1981, 1983]. Moreover, it was revealed that special granular inclusions of erythrocytes containing hematin are involved in neutralizing increased concentrations of sulfides [Holden et al., 1994; Vismann, 1993]. We demonstrated the role of these inclusions in H_2S neutralization for *A. kagoshimensis* [Soldatov et al., 2018]. The present paper provides material in the development of these patterns.

The aim of the work is to study the effect of increased concentrations of hydrogen sulfide on morphological and morphometric characteristics of erythroid elements of *A. kagoshimensis* hemolymph under experimental conditions.

MATERIAL AND METHODS

The work was carried out on adult molluscs sampled in June 2021 in the Laspi Bay waters (the Crimea). The shell height (from the hinge to the valve edge) ranged within 26–38 mm.

Experimental design. Molluscs of the control group were kept in an aquarium with oxygen concentration of 7.0–7.1 mg $O_2 \cdot L^{-1}$ (normoxia). *A. kagoshimensis* of the experimental group were exposed to hydrogen sulfide loading created by Na_2S donor dissolving in water to a final concentration of 6 mg $S^{2-} \cdot L^{-1}$. The exposure lasted 24 h (the first day of the experiment). The presence of sulfide ion in water resulted in its alkalization. This was compensated by adding 0.1 n HCl. The values of pH were maintained at 8.20–8.27. Sulfide ion interacted with oxygen, and it was accompanied by a decrease in the content of both gases in the aquarium water over time. After 24 h, the oxygen level in water amounted to 1.8 mg $O_2 \cdot L^{-1}$, and hydrogen sulfide was not detected. From seven individuals, hemolymph was sampled from the extrapallial space. The other seven individuals were subjected to repeated hydrogen sulfide loading. Na_2S was added to the aquarium water to a final concentration of 9 mg $S^{2-} \cdot L^{-1}$. After 24 h (the second day of the experiment), trace oxygen concentration, 0.03 mg $O_2 \cdot L^{-1}$, was registered in the aquarium water, and the level of hydrogen sulfide was of 1.9 mg $S^{2-} \cdot L^{-1}$. Hemolymph was sampled from these molluscs as well.

The oxygen content in water was monitored using a dissolved oxygen meter ST300D (Ohaus, the USA). The values of pH were measured on an InoLab pH 720 laboratory meter (Germany). The amount of sulfide ion in water was determined potentiometrically using a sulfide-selective sensor MSBS (the Netherlands).

Morphometric characteristics of erythrocytes. Smears were stained according to Pappenheim and analyzed under a light microscope Biomed PR2 LYuM equipped with a Levenhuk C NG Series camera. Cell diameter (C_1 and C_2) and nucleus diameter (N_1 and N_2) were measured from photographs in ImageJ 1.44p program (Fig. 1). On each smear, the indicated values were determined for 100 cells. Based on the obtained values, we calculated the mean cell volume (V_c) [Houchin et al., 1958], nucleus volume (V_n), cell thickness (h) [Chizhevsky, 1959], cell surface area (S_c) [Houchin et al., 1958], specific cell surface area (SS_c), and nuclear-cytoplasmic ratio (NCR) applying known algorithms:

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

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

$$h = 1.8 + 0.0915 \cdot (C_1 - 7.5) \,,$$

$$S_c = 2\pi a^2 b + \frac{2\pi a b sin(h^{-1}e)}{e} \,,$$

where

$$e = \frac{\sqrt{a^2 - b^2}}{a} \,, \quad a = \frac{C_1 + C_2}{4} \,, \quad b = 0.67h \,, \quad SS_c = \frac{S_c}{V_c} \,, \quad NCR = \frac{V_n}{V_c} \,.$$

Simultaneously, the number of erythrocyte abnormalities was determined on hemolymph smears *per* 1,000 cells.

Statistical comparisons were performed using the nonparametric Mann–Whitney *U* test. The results are presented as ($M \pm m$). The standard Grapher package (version 11) was applied.

RESULTS

Morphometric characteristics. Erythrocytes in *A. kagoshimensis* hemolymph are large rounded cells (Fig. 1A). The longitudinal (C_1) and transverse (C_2) diameters have similar values: (18.86 ± 0.61) and (16.13 ± 0.52) μm, respectively. The mean cell volume (V_c) is (678.5 ± 52.0) μm^3, and the cell surface area (S_c) is (1,037.5 ± 78.4) μm^2. The nucleus is compact, with a high proportion of heterochromatin which reflects the low functional activity of this structure. The shape is ellipsoidal [N_1 of (5.46 ± 0.09) μm; N_2 of (4.11 ± 0.10) μm]. The nucleus is usually located in the cell center. The volume (V_n) is (50.1 ± 3.1) μm^3. The nuclear-cytoplasmic ratio (NCR) is low, 0.08, which also indicates suppressed function of the cell nucleus. The cytoplasm is acidophilic, with a high hemoglobin content and a large number of small granular inclusions.

On the first day, hydrogen sulfide loading was accompanied by a significant increase in the volume of the cell and its nucleus (Fig. 2). A rise amounted to 24.3 and 30.1%, respectively, and it was statistically significant ($p < 0.05$). It is clearly visible that the growth was close. This was evidenced by the fact that NCR values retained at the level of control ones. The cell surface area rose by almost 23% ($p < 0.05$) and reached (1,275.5 ± 99.6) μm^2. At the same time, the specific cell surface area (SS_c) did not change and averaged 1.53 μm^{-1}. The number of free hematin granules in the hemolymph increased.

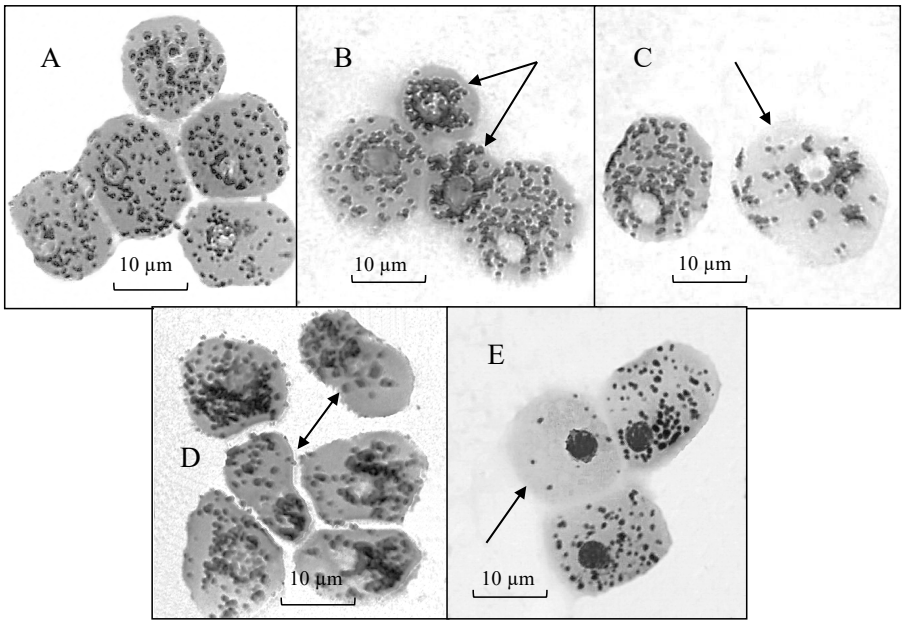

Fig. 1. Morphological types of cells in *Anadara kagoshimensis* hemolymph (A, normocytes; B, microcytes; C, macrocytes; D, cells with an altered shape; E, cells with a low number of granular inclusions)

On the second day of the experiment, the situation was the opposite. The cell volume decreased significantly (see Fig. 2): compared to control values, there was a drop by 36.4% ($p < 0.05$), and relative to the first day, by 48.9% ($p < 0.01$). The nucleus volume and erythrocyte surface area changed similarly. The changes were proportional which reflects the retention of NCR and SS_c values.

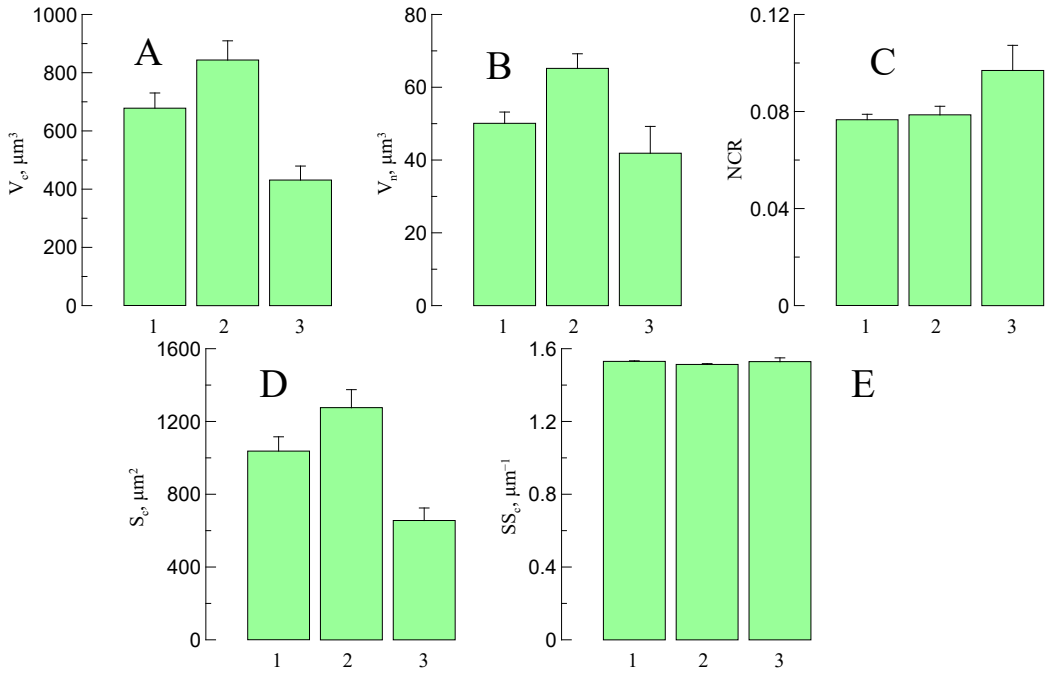

Fig. 2. Morphometric characteristics of erythroid elements of *Anadara kagoshimensis* hemolymph under conditions of hydrogen sulfide loading (A, V_c; B, V_n; C, NCR; D, S_c; E, SS_c; 1, the control group; 2, the first day of the experiment; 3, the second day of the experiment)

Morphological features. Analysis of the morphological features of erythroid cells showed a significant increase in the number of microcytes in the mollusc hemolymph under conditions of hydrogen sulfide loading (the first and second days of the experiment) (Fig. 1B, 3A). Those accounted for 6.6–7.0% of cells; it was almost three times higher than control values ($p < 0.05$). Microcytes were characterized by lower values of the transverse cell diameter (less than 15 μm). Interestingly, there was an increase in the number of macrocytes in the mollusc hemolymph on the first day by 30–32% ($p < 0.05$) (Fig. 3B). Their cell cross-section exceeded 22 μm (Fig. 1B).

Under conditions of hydrogen sulfide loading (the first day of the experiment), the number of erythrocyte abnormalities in the mollusc hemolymph rose. Cells with an altered shape and extremely low content of granular inclusions appeared (Fig. 1D, E). Their number increased by 30–50% relative to the control level (Fig. 3C, D). However, due to much individual variability in the obtained values, the differences were not statistically significant.

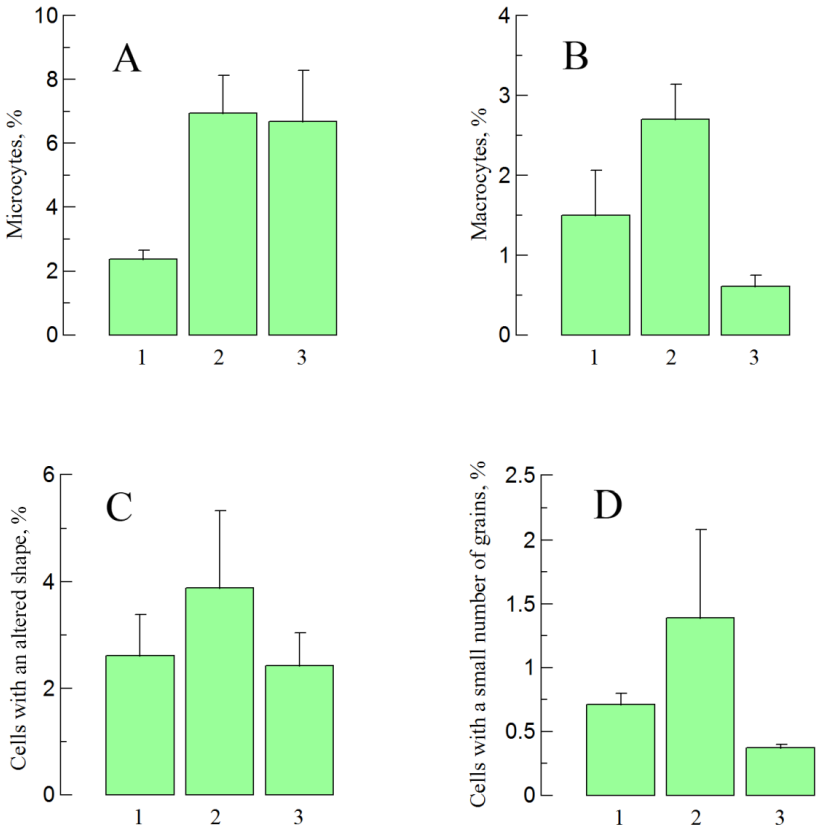

Fig. 3. Content of cells of various morphological types in *Anadara kagoshimensis* hemolymph under conditions of hydrogen sulfide loading (A, microcytes; B, macrocytes; C, cells with an altered shape; D, cells with a small number of grains; 1, the control group; 2, the first day of the experiment; 3, the second day of the experiment)

DISCUSSION

Double addition of Na_2S to the aquarium water where the molluscs were kept led to the development of an ambiguous situation:

- after the first day, moderate hypoxia occurred in water (1.8 mg $O_2 \cdot L^{-1}$), and hydrogen sulfide was not detected, which seems to be the consequence of the interaction of the latter one with oxygen;

- repeated addition of Na_2S (the second day) resulted in the formation of anoxia with retention of hydrogen sulfide contamination at the level of 1.9 mg $S^{2-} \cdot L^{-1}$.

The state of the erythroid population of the mollusc cells in each case had its own specifics.

The first day of the experiment. Compared to the control, the erythroid cell population became more heterogeneous. This was evidenced by a significant increase in the content of macro- and microcytes in the hemolymph, a rise in the number of cells with an altered shape, and a reduced number of granular inclusions in the cytoplasm. The mean cell volume increased by more than 20%. A rise in V_c can be determined by several processes.

Considering that the bivalves were kept under conditions of moderate hypoxia, we can assume the development of a swelling reaction in red blood cells, which is observed in many hydrobionts, including molluscs [Holk, 1996; Jensen et al., 1998; Nikinmaa et al., 1987; Novitskaja, Soldatov, 2011]. This reaction is believed to be aimed at correcting the intracellular pH value and determined by the work of the Na^+/H^+ antiporter [Tufts, 1992]. The swelling is controlled by catecholamines (adrenaline and norepinephrine) and is implemented *via* cell β-adrenergic receptors and cAMP [Ferguson, Boutilier, 1988; Salama, Nikinmaa, 1990; Val et al., 1997]. In our case, an increase in the content of catecholamines in the mollusc hemolymph can be expected, since the transition to conditions of moderate hypoxia occurred in a relatively short period of time. However, this process can determine a growth of cell volume by no more than 5–6% [Nikinmaa et al., 1987], and this is not entirely consistent with the values of changes in V_c provided in this work (> 20%). Even taking into account the higher elasticity of cell membranes of molluscs, indirectly evidenced by a wider range of osmotic resistance of their erythroid elements [Novitskaja, Soldatov, 2011], this increase can be considered as excessive.

Interestingly, there was a rise in the number of macrocytes in *A. kagoshimensis* hemolymph with the diameter exceeding 22 μm. A gain in the level of these cellular forms may explain such an increase in the mean cell volume (V_c). Their occurrence in a mollusc hemolymph usually precedes the apoptosis, when a cell disintegrates into separate fragments (apoptotic bodies) [Manskikh, 2007]. In the case of *A. kagoshimensis*, this reaction has the meaning of adaptation [Soldatov et al., 2018]: when a cell is destroyed, hematin-containing granular inclusions are released in a significant amount into the hemolymph [Holden et al., 1994; Vismann, 1993]. Hematins have a high oxidizing ability and can interact with hydrogen sulfide [Vismann, 1993]. The most likely product of this interaction is ferric sulfide:

$$2Fe^{3+} + 3S^{2-} \rightarrow Fe_2S_3 \ .$$

It is an unstable compound that, in the presence of oxygen, is oxidized to ferric oxide releasing atomic sulfur:

$$2Fe_2S_3 + 3O_2 = Fe_2O_3 + 6S^0 \ .$$

As known, some species of marine invertebrates are capable of accumulating sulfur under conditions of hydrogen sulfide contamination [Powell et al., 1980]. This allows us to assume the sequence of events discussed above. A rise in the content of erythrocytes with a reduced number of granular inclusions in *A. kagoshimensis* hemolymph under conditions of hydrogen sulfide loading also gives us the grounds to make an assumption on the ability of these cells to remove hematin granules beyond their membranes not violating their integrity.

The second day of the experiment. Distinctive features of the population of erythroid elements of *A. kagoshimensis* hemolymph on the second day were the high content of microcytes in it and a significant decrease in the mean cell volume (V_c). Apparently, the latter was determined by a drop in the number of macrocytes in the mollusc hemolymph, since the content of microcytes on the first and second days of the experiment was close. An increase in the number of microcytes in the hemolymph may be due to several processes.

The first process is fragmentation of the cytoplasm areas of red blood cells leading to the formation of schistocytes. In this case, the cell size decreases (microcytes are formed). This phenomenon was noted for organisms at various levels of organization, including humans [Bessman, 1988]. It is usually observed during the development of anemia states. It was also shown for *A. kagoshimensis* under conditions of external anoxia [Soldatov et al., 2021]. Apparently, this is the main process that results in the formation of a large number of microcytes in the mollusc hemolymph in a relatively short time.

The process close to fragmentation is direct division of highly specialized cells (amitosis), *inter alia* erythrocytes. It involves a random distribution of nucleus material [Fuller, Shields, 1998]. A particular manifestation of this process is the formation of anucleate cells and microcytes. It occurs if the nucleus, *prior* to cytokinesis (formation of a constriction), moves towards one of the cell poles. This phenomenon was described for *A. kagoshimensis* as well [Novitskaya, Soldatov, 2013]. It can also be observed in cells with an altered shape in the present work (Fig. 1D).

The formation of microcytes is also possible during intensive hematopoiesis (erythropoiesis); it is most often observed under conditions of oxygen deficiency similar to those for *A. kagoshimensis*. However, data on this issue in relation to molluscs are extremely scarce [Furuta, Yamaguchi, 2001], which does not allow us to accept this interpretation as a basis.

Conclusion. Under conditions of short-term hydrogen sulfide loading (the first day), the population of *Anadara kagoshimensis* erythroid elements becomes more heterogeneous. In the hemolymph, the content of micro- and macrocytes increases, and the number of cells with an altered shape and low content of granular inclusions in the cytoplasm rises. The number of free hematin granules in the mollusc hemolymph increases noticeably. The mean cell volume (V_c) rises by more than 20%. Staying under conditions of increased sulfide concentration within the second day results in a significant decrease in V_c, which is determined by a notable reduction in the population of macrocytes in *A. kagoshimensis* hemolymph.

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МОРФОМЕТРИЧЕСКИЕ ХАРАКТЕРИСТИКИ ЭРИТРОИДНЫХ ЭЛЕМЕНТОВ ГЕМОЛИМФЫ *ANADARA KAGOSHIMENSIS* (TOKUNAGA, 1906) В УСЛОВИЯХ СЕРОВОДОРОДНОГО ЗАРАЖЕНИЯ

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В условиях эксперимента исследовали влияние сероводородной нагрузки на морфометрические характеристики эритроидных элементов гемолимфы *Anadara kagoshimensis* (Tokunaga, 1906). Работа выполнена на взрослых особях моллюска с высотой раковины 26–38 мм. Контрольную группу моллюсков содержали в аквариуме с концентрацией кислорода 7,0–7,1 мг $O_2 \cdot л^{-1}$ (нормоксия). Экспериментальную группу подвергали действию сероводородной нагрузки, создававшейся при растворении в воде донора Na_2S до финальной концентрации 6 мг $S^{2-} \cdot л^{-1}$. Спустя сутки уровень кислорода в воде составил 1,8 мг $O_2 \cdot л^{-1}$, а сероводород не был обнаружен. Часть моллюсков подвергали повторной сероводородной нагрузке путём внесения Na_2S до финальной концентрации 9 мг $S^{2-} \cdot л^{-1}$. К концу вторых суток в воде регистрировали 1,9 мг $S^{2-} \cdot л^{-1}$ и следовую концентрацию кислорода — 0,03 мг $O_2 \cdot л^{-1}$. В условиях краткосрочной сероводородной нагрузки (первые сутки) популяция эритроидных элементов анадары становилась более гетерогенной. В гемолимфе повышалось содержание микро- и макроцитов, увеличивалось число клеток с изменённой формой и низким содержанием зернистых включений в цитоплазме. Число свободных гранул гематина в гемолимфе существенно росло. Среднеклеточный объём (V_c) увеличивался более чем на 20 %. Пребывание в условиях повышенной концентрации сульфидов в течение двух суток приводило к значительному понижению V_c, что определяется существенным сокращением популяции макроцитов в гемолимфе моллюсков.

Ключевые слова: моллюски, *Anadara kagoshimensis*, сероводород, гемолимфа, эритроидные элементы