ORIGINAL PAPERS

Erythroid Elements of Hemolymph in *Anadara kagoshimensis* (Tokunaga, 1906) under Conditions of the Combined Action of Hypoxia and Hydrogen Sulfide Contamination

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Received May 31, 2018

Abstract—The effect of the combined action of hypoxia (0.05 mg O_2/L) and hydrogen sulfide contamination (4–8 mg S^{2-}/L) on the morphometric characteristics of the bivalve mollusk *Anadara kagoshimensis* (Tokunaga, 1906) was studied under experimental conditions in vivo; the exposure lasted for 24 hours. A significant increase in the size of granular inclusions in cells was observed. The number of these granular inclusions increased by 72–178%, in comparison with the control values (normoxia). The volume of erythrocytes increased by 15–74% and reached 608.1 ± 15.2 μm³ in some individuals. The cells formed aggregates of different shapes and sizes against the background of pronounced poikilocytosis. A significant increase in the number of erythrocyte shadows became a mass phenomenon. Simultaneously, granular inclusions containing hematin entered into the hemolymph of the mollusk. It is assumed that these granular inclusions reacted with H_2S to form Fe_2S_3 and thus neutralized the toxic effect of hydrogen sulfide.

INTRODUCTION

The low rate of oxygen diffusion in the aquatic environment and the restriction of water exchange in marine areas are the main causes of the formation of hypoxic and anoxic zones. Most often, these phenomena are observed in the bottom layers of the water column and are combined with a local increase in the concentration of hydrogen sulphide [2]. Benthic organisms living in such conditions must have tolerance to these factors

Adaptive reorganization of tissue metabolism in mollusks under conditions of hypoxia (anoxia) has been well studied [14, 15]. However, the resistance of these animals to hydrogen sulfide pollution is not completely clear. A number of studies have noted the presence of symbiosis with sulfide-oxidizing bacteria, which massively settle on the respiratory surfaces of mollusks [25]. The presence of a special transport protein in the hemolymph, the appearance of hemoglobins insensitive to H_2S [7, 8], and other effects have also been discussed. In the experiments performed on the ark clam *Anadara kagoshimensis*, attention was paid to the stability of the mollusk to hypoxia and anoxia [18, 23] and to the ability of the mollusk to cope with the presence of H_2S in water [19, 20]. Even at an

extremely low oxygen concentration (less than 1.2% saturation) the ark clam did not reduce the oxygen consumption rate [10] and the highest energy conservation was noted under the conditions of anoxia. This allows the mollusk to survive for a long period of time under the conditions of extreme hypoxia and anoxia [[27]. Particular attention was paid to the presence of special granular inclusions in the erythroid cells of *Anadara sp.* clams, whose nature has been studied for more than 120 years [11]. It was shown that these inclusions are characteristic of erythrocytes not only in *Anadara sp.,* but also in a number of other marine invertebrates [8, 13]. The results of electron microscopy reveal that granular inclusions are round relatively homogeneous formations that do not have any internal organization [16]. Histochemical studies reveal peroxidase and weak lysosomal activity and the presence of iron, fats, and vitamin C in the inclusions [16]. The authors suggest that these inclusions contain a unique heme that is a part of hematin, which is used as an iron reserve in the synthesis of hemoglobin [16, 30]. Moreover, the ability of the mollusk to neutralize elevated concentrations of sulfides is associated with hematin [30].

In the Black Sea, the population of *Anadara* sp. inhabits primarily hypoxic ecotopes that are periodi-

cally exposed to hydrogen sulfide contamination [4]. The clam individuals are characterized by a highly effective antioxidant enzyme complex and a specific spectrum of carotenoids in tissues [24]. The morphological features of the erythroid cells of the hemolymph of the Black Sea clams have been described; these are similar in different representatives of the genus *Anadara* [21], and in their variation under experimental anoxia [3].

In the present work, the morphological characteristics of the erythroid cells in hemolymph of the ark clam, *Anadara kagoshimensis,* have been studied under the combined effect to extreme hypoxia and hydrogen sulfide loading.

MATERIALS AND METHODS

Anadara kagoshimensis specimens with a shell length of 30−33 mm were used in the study. The mollusks were transported in a container in bulk without water for 1 h from the time of collection. Before the experiment, mollusks were kept for 2−3 days in aquariums with flowing sea water to relieve stress.

The experimental study was made on a specially designed stand. Five clam specimens were placed in a 4.5-liter chamber. The oxygen content in water was reduced from 8.5−8.7 to 0.05 mg O_2/L by pumping water with N_2. The oxygen concentration was monitored potentiometrically. A DO Meter ST300D RU oximeter (Ohaus, United States) was used in the work. A mother solution of Na_2S was then added into the chamber to a final concentration of 4−8 mg S^{2-}/L. The HS^{2-} value in the water was determined potentiometrically using an MSBS sulfide-selective sensor (Netherlands). Dissolution of Na_2S in seawater is accompanied by an increase in pH; therefore, to compensate for these changes, 1 N HCl solution was added into the chamber. The pH values were measured with an InoLab pH 720 pH meter (Germany).

The water temperature during the test was maintained at $20 \pm 1°C$, a photoperiod of 12-h day : 12 h night, and an exposure of 24 h. The control group of mollusks was kept under similar conditions at an oxygen concentration in water of 8.5−8.7 mg O_2/L (95−97% saturation).

Hemolymph samples were obtained by puncturing the extrapallial cavity with a syringe immediately after the experiment was completed. Before the preparation of smears for cytological studies, the erythroid mass was washed three times in isotonic NaCl solution (0.85%) and centrifuged at 3500 rpm for 15 min to remove the salts that actively crystallize when the smear dries.

The prepared smears were stained using the combined Pappenheim method (May-Grunwald + Romanowski-Gimza). The smears were studied for morphological and cytometric characteristics of erythroid elements. A Biolar light-optical microscope (Poland) was used in the work.

The linear dimensions of the blood cells were determined from photographs in the ImageJ 1.44p computer program [12]. The major and minor axes of the cell (C_1 and C_2) and their nuclei (N_1 and N_2) were measured; the selected aggregation was 100 cells per smear. The volume of an erythrocyte (V_c) [17] in terms of the volume of its nucleus (V_n) [5] and cell thickness (h) [6] was determined by the equations:

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

$$V_n = \frac{\pi N_1 N_2^2}{6}; \quad h = 1.8 + 0.0915(C_1 - 7.5).$$

The nucleo-cytoplasmic ratio (NCR) was calculated as the ratio of the volume of the cell nucleus (V_n) to its volume (V_c). Statistical processing and graphic display of the results were performed using the Grapher standard package (version 7). The results are presented in this form. The reliability of the differences was assessed using the Student's *t*-test. The normality of the distribution was judged by the Pearson criterion. The number of animals used in the study is given in Table 1.

RESULTS

Normoxia (8.5−8.7 mg O_2/L). The erythroid elements of the hemolymph are slightly ellipsoidal in shape (Fig. 1a). In the control group of individuals, the large (C_1) and the small (C_2) cell diameters were similar in dimension (Table 1). The cell volume was 350.5 ± 6.7 μm^3. The acidophilic cytoplasm, which reflected the presence of hemoglobin, contained black granular inclusions at 21.8 ± 0.7 per cell, on average. The smears included erythrocyte shadows; their quantity did not exceed 14% of the total cell weight. The cells contained a small ellipsoidal nucleus (Table 1), the nucleus volume, as calculated from the ellipsoid rotation formula, was 19.3 ± 1.9 μm^3. The nuclear content was compact, with highly concentrated chromatin; it was sharply basophilic in color reflecting the low functional activity of the structure. This was also shown by lower NCR values.

Hypoxia (0.05 mg O_2/L) was accompanied by a slight increase in the linear dimensions of red blood cells and their nuclei (Fig. 1b). The longitudinal and cross sections of erythrocytes increased by 5 and 8% ($p < 0.05$) relative to the control group of mollusks, respectively. Cells became more rounded (Table 1); their volume increased by 10% and reached 385.1 ± 11.3 μm^3. However, the differences were not statistically significant. The number of granular inclusions in the cytoplasm increased by 37% ($p < 0.001$). The number of erythrocyte shadows was 4.2 times higher than in the hemolymph of the control group of mollusks. In

Table 1. The morphometric characteristics of erythroid cells of the *Anadara* hemolymph under conditions of normoxia, hypoxia, and hypoxia in combination with a hydrogen sulfide load

Index	Experimental Conditions		
	normoxia (8.5−8.7 mg O_2/L)	hypoxia (0.05 mg O_2/L)	hypoxia + H_2S (0.05 mg O_2/L; 4−8 mg S^{2-}/L)
C_1, μm	11.3 ± 0.3(10)	11.9 ± 0.1(12)	17.9 ± 0.6(6)
C_2, μm	10.2 ± 0.3(10)	11.0 ± 0.1(12)	14.1 ± 0.4(6)
N_1, μm	3.79 ± 0.09(10)	4.11 ± 0.05(12)	3.96 ± 0.13(6)
N_2, μm	3.09 ± 0.11(10)	3.20 ± 0.03(12)	3.50 ± 0.16(6)
V_c, μm^3	350.5 ± 6.7(10)	385.1 ± 11.3(12)	484.2 ± 22.4(6)
V_n, μm^3	19.3 ± 1.9(10)	23.6 ± 0.8(12)	31.4 ± 2.4(6)
NCR	0.055 ± 0.002(10)	0.061 ± 0.002(12)	0.062 ± 0.003(6)
Number of grains/units	21.8 ± 0.7(10)	29.9 ± 0.6(12)	49.0 ± 4.4(6)

In parentheses, the number of individuals; C_1, large, C_2, small cell diameters; N_1, large nucleus diameter, N_2, small nucleus diameter; V_c, cell volume; V_n, nucleus volume; NCR, nucleo-cytoplasmic ratio.

parallel, the linear dimensions of the cell nuclei increased by 3−8%. A noticeable increase in the volume of the nucleus occurred, which reached 22% ($p < 0.05$). In the cell population, the number of erythroid elements with a core volume of 20−30 μm increased; the increase in the size of the nucleus was proportional to the growth of the cell volume. NCR values did not change (Table 1). The differences were not statistically significant.

Combination of hypoxia with hydrogen sulfide load (0.05 mg O_2/L; 4−8 mg S^{2-}/L). This combination of factors was accompanied by significant morphological changes in the erythroid elements of the hemolymph (Table 1). The cell volume increased by 15−74% and reached 608.1 ± 15.2 μm^3 in some individuals. The number of granules (72−178% relative to the control value) increased and their size grew (Fig. 2a). Against the background of pronounced poikilocytosis (Fig. 2b) the cells formed aggregates that were different in shape and size. The number of erythrocyte shadows significantly increased and their presence became a mass phenomenon (Fig. 2c). Against this background, granular cell inclusions were released into the hemolymph (Fig. 2d). The inclusions changed their color; this might indicate chemical transformations.
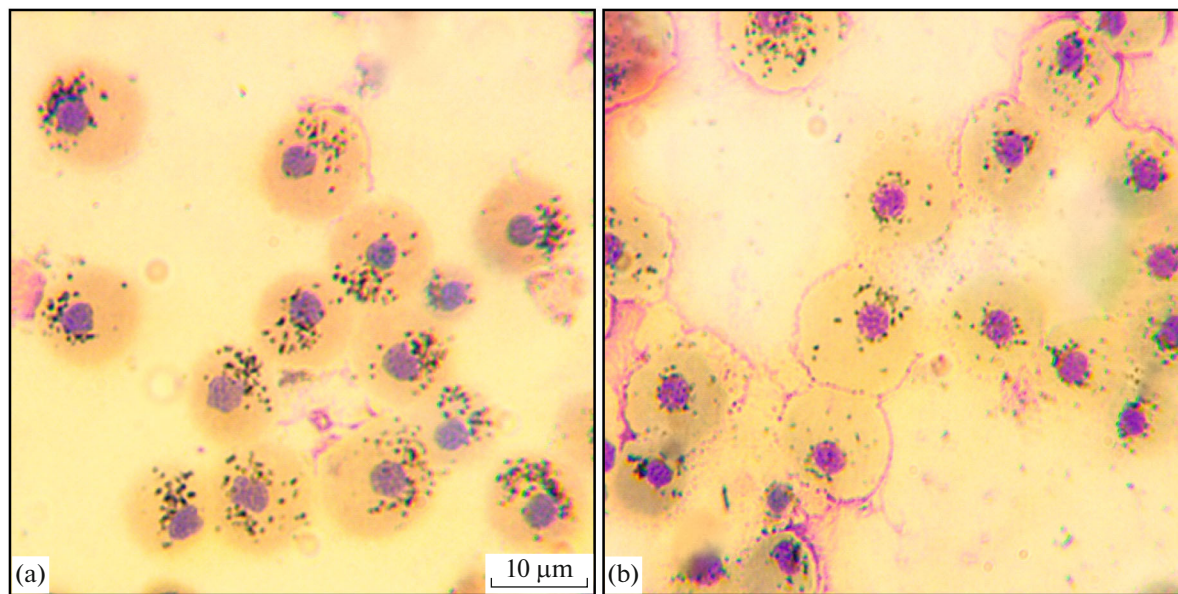
Fig. 1. The erythroid cells of the *Anadara* hemolymph under conditions of normoxia (a) and hypoxia (b).
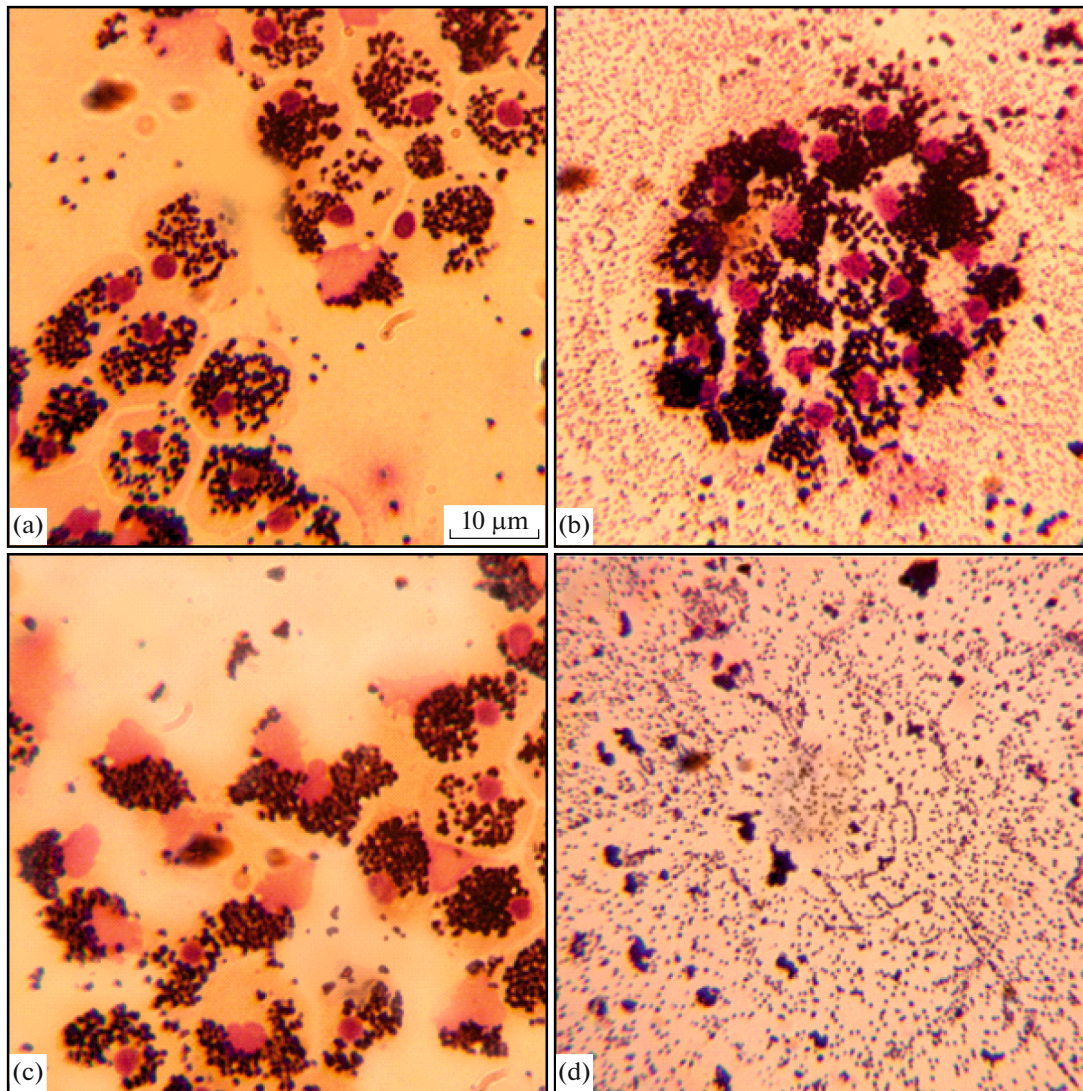
Fig. 2. The erythroid cells of the *Anadara* hemolymph under conditions of combined action of hypoxia and a hydrogen sulfide load. (a) Individual cells; (b) aggregates; (c) erythrocyte shadows; (d) free granules.

DISCUSSION

The increase in the number of granular inclusions in the cell cytoplasm with an oxygen deficit seems to be relative in our case. Earlier it was shown that under conditions of hypoxia (0.05 mg O_2/L) in this type of hemolymph the number of cells of late generations with a low content of granular inclusions decreased [3]. This occurred against the background of an increase in the osmotic resistance of the erythrocyte mass, which suggests selective lysis of the old red blood cells. Apparently, this process also occurred in our case.

Under the combined action of hypoxia and a hydrogen sulfide load we observed a significant increase in the number and size of granular inclusions in the erythroid elements of the *Anadara* hemolymph. We hypothesize that granular inclusions in the erythrocytes of the clam contain a unique heme that is a part of hematin [16, 26]. Hematins are an oxidized form of heme, they occur during hydrolysis of oxyhemoglobin. Hematins have the form of dark brown (black) rhomboid crystals (Teichmann's crystals) or grains; they give birefringence in polarized light (anisotropy) and contain iron in a trivalent state [1]. Hematins have high oxidizing ability and can interact with hydrogen sulfide [26]. The most probable product of this interaction is ferric sulphide: $2Fe^{3+} + 3S^{2-} \rightarrow Fe_2S^3$. This is an unstable compound, which is oxidized in the presence of oxygen to ferric oxide with the release of atomic sulfur:

$$2Fe_2S_3 + 3O_2 = Fe_2O_3 + 6S°.$$
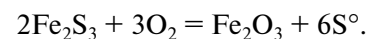
The ability of certain species of marine invertebrates to accumulate sulfur under hydrogen sulfide contamination is known [22]; this allows us to accept the order of events discussed above. Of course, this

process is aimed at compensation of the hydrogen sulfide load; however, it can be effective only in the presence of low concentrations of oxygen, which will allow the conversion of Fe_2S_3 to Fe_2O_3 with the liberation of S^o. Under the experimental conditions, we intentionally used rather high concentrations of hydrogen sulfide ($4-8$ mg S^{2-}/L), which are typical of the depths of $1200-1500$ m in the Black Sea. In the coastal zone of hypoxia (anoxia) its concentrations are significantly lower and the reactions noted above will have an adaptive rather than compensatory significance.

Thus, the combined effect of hypoxia and hydrogen sulfide loading led to an increase in the size and number of granular inclusions in the erythrocytes of *Anadara* species and to the growth of the cell volume. Against the background of pronounced poikilocytosis, the hemolymph cells formed aggregates that are different in shape and size. The number of erythrocyte shadows significantly increased in smears. Simultaneously, there was a flow into the hemolymph of granular inclusions that contained hematin.

ACKNOWLEDGMENTS

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Translated by I. Barsegov