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EXPERIMENTAL PAPERS

Effect of Ranged Short-Term Hypoxia on Functional and Morphological Parameters of Hemocytes in the Pacific Oyster *Crassostrea gigas* (Thunberg, 1793)

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Abstract—The effect of ranged short-term (24 h) hypoxia on morphological and functional parameters of hemocytes in the Pacific oyster (*Crassostrea gigas*) were investigated using flow cytometry and light microscopy. The control group was kept at 100% oxygen saturation, experimental groups were exposed to moderate (30% oxygen saturation) and severe hypoxia (3% oxygen saturation). Hypoxia had no effect on morphometric parameters of hemocytes, but induced considerable changes in their functional characteristics, leading to shifts in the cellular composition of hemolymph. In the oysters exposed to moderate oxygen deficiency, a compensatory response consisted in an increase in the granulocyte count (by 20%) and an increase in the spontaneous production of reactive oxygen species in agranulocytes (by 40%) and granulocytes (by 90%). Severe short-term hypoxia inhibited the ability of hemocytes to generate an oxidative burst and induced a decrease in the granulocyte count, indicating the inability of oysters to maintain normal functional state.

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INTRODUCTION

Oxygen deficiency is one of the most significant environmental factors that influence the vital activity of aquatic organisms [1]. In the littoral and sublittoral zones, hypoxia can ensue due to natural cycles of fluctuations in dissolved oxygen levels or eutrophication [2]. The duration of hypoxic exposure under natural conditions can vary from several hours to several months. Persistent hypoxia is considered to be the main reason for the decline in the biodiversity of aquatic habitats [3]. Short-term hypoxia, in turn, can negatively affect the functional state of aquatic organisms. Benthic and sedentary aquatic species, including bivalve mollusks, are particularly susceptible to hypoxia [4]. It should be noted that many bivalvian species are intensively cultivated all over the world, gaining not only biological, but also economic significance [5]. The Pacific oyster (*Crassostrea gigas*, Thunberg 1973) is considered one of the main worldwide objects of aquaculture

because of its high growth rate, euryhalinity, high adaptive potential toward oxygen deficiency and temperature fluctuations [6]. The optimal oxygen regime for aquatic organisms, specifically for bivalve mollusks, is the dissolved oxygen concentration in water at a level of 7.5–9 mg O_2/L. However, *C. gigas* often inhabits shallow coastal areas characterized by eutrophication and poor water mixing, due to which such areas may become hypoxic [7, 8, 9]. It was noted that with a decrease in the oxygen concentration below 3 mg O_2/L, in the oyster organism, oysters reveal various physiological disorders, low resistance to bacterial pathogens, as well as reduced growth rate and survival rate of individual mollusks [10, 11, 12, 13, 14].

In the Black Sea, the Pacific oyster has been cultivated for more than 40 years [15]. For farmed oyster species, a deeper insight into the mechanisms and consequences of the impact of natural environmental factors on the immune system is of great importance. The latter arouses a high interest in studying the influence of environmental stress factors on the ability of the immune system to resist infectious agents [16]. Hemocytes circulating in the mollusk hemolymph are considered to be the main cell type that reflects the physiological status of an organism due to their extensive functional role, specifically the involvement in shell regeneration, digestion, transport of nutrients, and protective immune responses [16, 17, 18, 19].

The cellular immune response in mollusks includes phagocytosis, encapsulation, enzymatic destruction of pathogens, and generation of reactive oxygen species (ROS) [20]. The humoral component of the immune system in bivalve mollusks is based on the production of C-type lectins, antimicrobial and peptidoglycan-recognizing proteins, and some other compounds [21, 22, 23]. Many works have been devoted to the impact of hypoxia on the functional state of hemocytes and their ability to generate an immune response. For example, the incubation of *Perna viridis* under conditions of oxygen deficiency elicits a decrease in ROS production [23, 24]. On the other hand, in *Mytilus galloprovincialis*, *Mytilus coruscus* and *Chlamys farreri*, short-term hypoxia leads to an increase in this parameter [25, 26, 27]. It is well known that oxygen deficiency leads to a decrease in the total hemocyte count [24, 25, 28, 29] and a change in their ratio in hemolymph [25, 26]. The ratio of hemocyte types is considered as an indicator of the effectiveness of the cellular immune status of an organism, since granular hemocytes are more capable of eliciting an immune response compared to their agranular counterparts [30]. At the same time, the proportion of granular hemocytes in *M. coruscus* decreased after incubation at 2 mg O_2/L [25], while in *M. galloprovincialis*, this parameter increased after a daily incubation at 0.3 mg O_2/L [26]. Based on the above data, it can be assumed that the hypoxic effect, depending on the degree and duration, can exert both a stimulatory and an inhibitory effect on the cellular immune component in mollusks. The boundaries of the adaptive potential to oxygen deficiency is especially important to assess for mass aquaculture objects, since during the production cycle, e.g., transportation, sizing and redistribution across oyster banks, mollusks often face short-term oxygen deficiency, which may last up to 24 h. At the same time, a short-term but deep hypoxic exposure can transcend the adaptive potential of cultivated objects.

The goal of this study was to explore the effect of ranged short-term hypoxia on morphological and functional characteristics of hemolymph cell components in a mass aquaculture object, the bivalve Pacific oyster *Crassostrea gigas* (Thunberg, 1793).

MATERIALS AND METHODS

Pacific oysters *C. gigas* (n = 30, weight 8.6 ± 0.4 g, shell length 25.4 ± 1.4 mm) were obtained from an oyster-mussel farm (Sevastopol Bay, Sevastopol). To adapt to laboratory conditions and relieve transportation-induced stress, oysters were kept for a week in containers with running seawater (oxygen content 7.9 mg/L, i.e. 100% oxygen saturation of water; temperature 22°C, salinity 19.5 ‰, pH 8.1 ± 0.01). During acclimation, the mollusks were fed with a mixture of microalgae (5–10 mL/50 L of aquarium water; cell concentration $2–3 \times 10^6$/mL). The control group of mollusks (n = 10) was kept at an oxygen concentration of 7.9 mg/L. Hypoxia was created

in vivo by blowing gaseous nitrogen through seawater for 1.5–2 h until a dissolved oxygen concentration of 30% (2.4 mg/L; $n = 10$) and 3% (0.2 mg/L; $n = 10$) versus the reference level. Upon reaching a desired level of hypoxic exposure, the mollusks were kept in oxygen-deficient water for 1 day. The dissolved oxygen concentration in experimental and control aquaria was monitored using a Starter 300D portable dissolved oxygen meter (Ohaus, USA) with a temperature sensor. The constancy of the dissolved oxygen concentration in experimental aquaria was achieved by a periodic water aeration. Salinity and pH were monitored using an ST20S salt meter (Ohaus, USA) and ST2100-F pH meter (Ohaus, USA). The pH of seawater, salinity, and temperature were identical in both control and experimental groups and corresponded to the acclimation period.

At the end of 24-h hypoxic exposure, hemolymph was collected. Hemolymph samples (0.5–1 mL) were taken from the heart via a sterile syringe and washed thrice in sterile filtered seawater (300 g, 5 min). Samples obtained from the same mollusk were analyzed individually. The morphometric characteristics of hemocytes sedimented by centrifugation were assessed on smears stained using the Pappenheim method [31]. At least 1000 cells were analyzed per smear. In each hemocyte, the morphological parameters were assessed, and the largest cell and nucleus diameters were measured (disregarding the pseudopodia). The nucleus-to-cytoplasmic ratio was calculated as the ratio of the nucleus diameter to the cell diameter [32, 33]. The functional characteristics of hemocytes were analyzed using flow cytometry (FC500 cytometer, Beckman Coulter) and Flowing Software 5.2. To assess the DNA content and proliferative cell activity, hemocytes were stained with SYBR Green I Dye (Sigma Aldrich) as described previously [34]. The types of hemocytes were identified and their percentage versus the total cell count in the suspension were carried out on two-parameter cytograms based on the distribution of SYBR Green-positive particles by their relative size (forward scatter, FS) and granularity (lateral scatter, SS). The hemocyte mortality rate was determined using propidium iodide (PI, Sigma Aldrich), a membrane-impermeable fluorescent DNA stain [32]. The ability of hemocytes to spontaneously produce ROS was assessed by the fluorescence of 2-7-dichlorofluorescein diacetate (DCF-DA) dye (Merck, Germany) according to the standard staining protocol [32].

Statistical data processing was carried out using the RStudio version 4.0.5 software. The Kolmogorov–Smirnov test showed that the distribution of hemocyte dimensional characteristics disobeys a normal distribution. To analyze the significance of the hypothesis on the presence of differences between the samples of microscopic results, the Mann–Whitney U-test was used. The results of cytometric studies were analyzed using one-way ANOVA and the Tukey's test. The critical significance level was taken as 0.05. The results were presented as $S_x \pm$ SE (mean and the error of the mean).

RESULTS

Using light microscopy, three type of hemocytes were identified in the oyster hemolymph: agranulocytes, hyalinocytes, and granulocytes (Figs. 1a–1c). Agranulocytes are the smallest cells with a diameter of 8.9 ± 0.4 μm characterized by a predominantly round shape, high nucleus-to-cytoplasmic ratio (0.6 ± 0.03 a.u.), and the absence of cytoplasmic granular inclusions and pseudopodia.

The cells with a largest diameter, granulocytes, had a low nucleus-to-cytoplasmic ratio (0.4 ± 0.04 a.u.); their cytoplasm contained basophilic and eosinophilic granular inclusions, and the nuclei were mainly located excentrically. Besides, granular cells formed pseudopodia. The diameter of hyalinocytes was 10.2 ± 0.8 μm, the diameter of their nuclei was 5.7 ± 0.4 μm. Hyalinocytes, like granulocytes, had pseudopodia, but their cytoplasm lacked granular inclusions, while the nucleus was mainly centrally located. The fluorescence peak of a DNA-specific dye SYBR Green I in control and experimental groups corresponded to a diploid set of chromosomes without signs of division (Fig. 2a). Using flow cytometry and based on the distribution of particles by forward (FS) and side (SS) scattering, 3 subpopulations of cells with different relative size and granularity

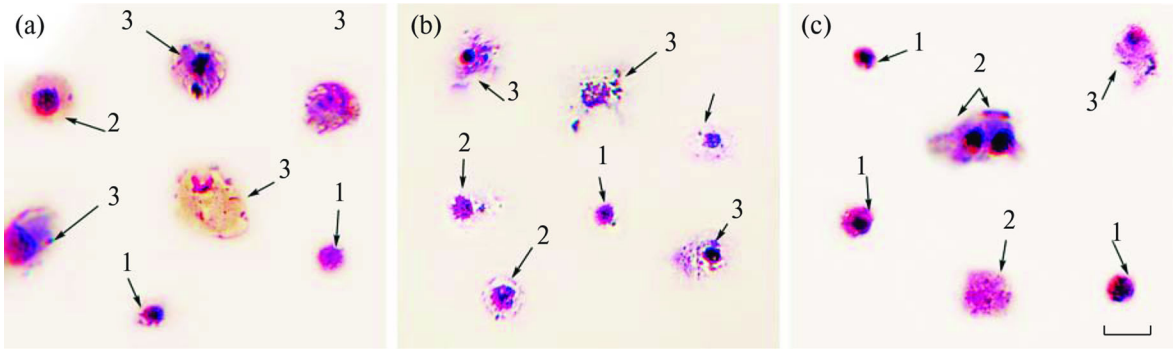

Fig. 1. Morphological characteristics of hemocytes in the Pacific oyster *Crassostrea gigas* under normoxic and hypoxic conditions. (a) Normoxia. (b) 30% oxygen saturation of water. (c) 3% oxygen saturation of water. 1—agranulocytes, 2—hyalinocytes, 3—granulocytes. Smears stained using a combined Pappenheim method were viewed under a light microscope (Biomed PR-2 Lum) equipped with a camera (Levenhuk C NG Series). Scale bar, 10 μm.

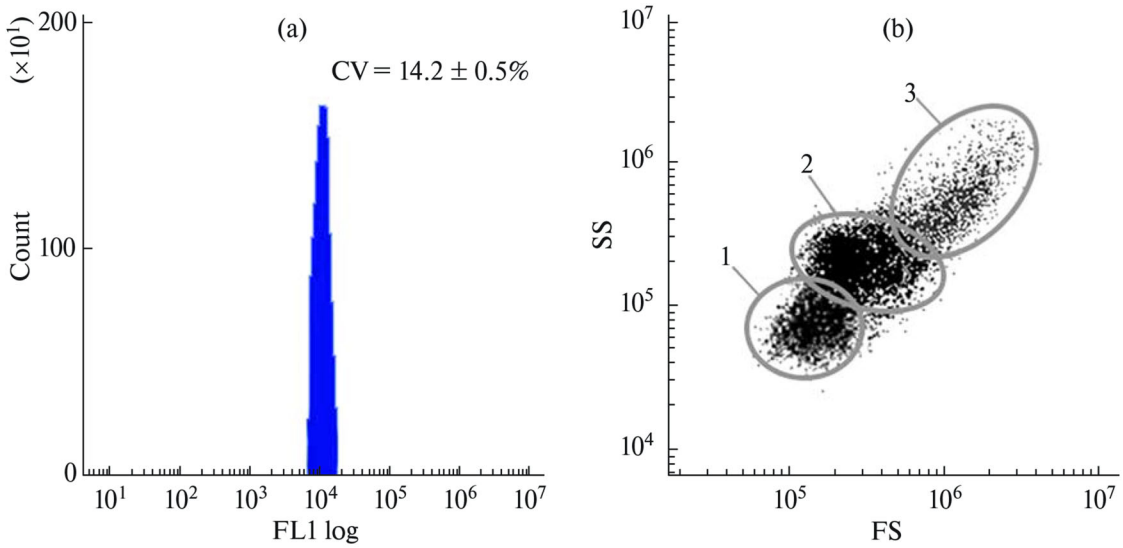

Fig. 2. Subpopulations of hemocytes in the Pacific oyster as identified by flow cytometry. (a) DNA content. Hemocytes were suspended in sterile filtered seawater (cell concentration $1–2 \times 10^6/mL^{-1}$), incubated with SYBR Green I for 40 min in the dark (final dye concentration in the smple 10 μM). CV—Coefficient of variation. (b) Hemocyte distribution based on relative size (forward scatter, FS) against granularity (side scatter, SS) values to reveal three hemocyte subpopulations: 1—agranulocytes, 2—hyalinocytes, 3—granulocytes. The plots are shown for the control group of mollusks.

level were identified (Fig. 2b). Agranular cells, agranulocytes (37.5 ± 14.2%) and hyalinocytes (58.8 ± 15.7%), were predominant in the hemolymph of oysters. The relative granulocyte count in the control sample was 8.8 ± 4.6%. The classification of hemocytes is described in more detail in our previous work [35].

24-h hypoxia had no effect on the morphometric characteristics of oyster hemocytes, however, it significantly affected the ratio of hemocyte types in hemolymph (Fig. 3). Incubation under conditions of 30% oxygen saturation of water led to a significant increase in the relative granulocyte count (by 20%, $p < 0.05$) (Fig. 3c), while the percentage of agranulocytes and hyalinocytes remained intact. After one-day incubation under conditions of 3% oxygen level of that under normoxia, the relative agranulocyte count increased by 58% (Fig. 3a), while the hyalinocyte count decreased by 50% (Fig. 3b). The hemocyte mortality rate did not exceed 2% in both control and experimental groups. No hypoxia-induced changes in the proliferative activity of hemolymph cells were observed.

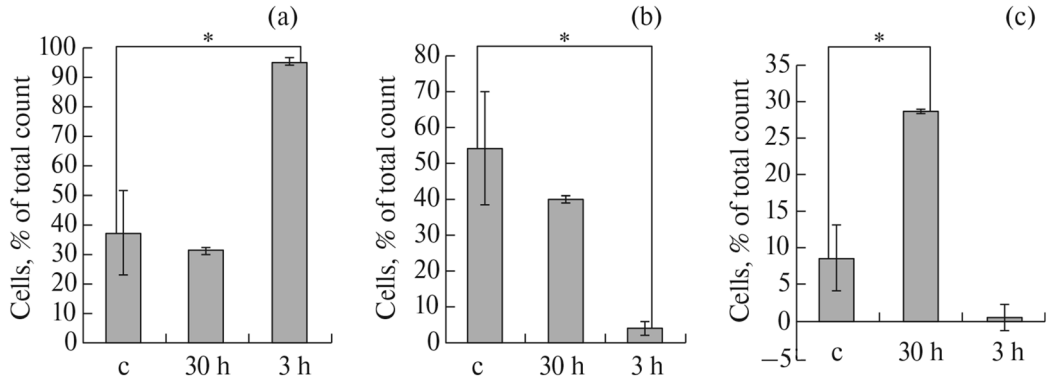

Fig. 3. Effect of short-term hypoxia on the ratio of hemocyte types in the Pacific oyster *Crassostrea gigas*. (a) Changes in the relative agranulocyte count. (b) Changes in the relative hyalinocyte count. (c) Changes in the relative granulocyte count. Mollusks were divided into three groups, which were held in water with different oxygen concentrations: c—control group, 100% oxygen saturation ($n = 10$); 30 h—experimental group, 30% oxygen saturation ($n = 10$); 3 h—experimental group, 3% oxygen saturation ($n = 10$). Hemocyte suspension was stained with a DNA dye SYBR Green I (final concentration in a sample, 10 μmol/L; incubation time, 40 min in the dark) to identify cell types in a Beckman Coulter FC500 flow cytometer. The ratio of cell types was assessed on the forward scatter vs. side scatter (FS/SS) histogram. *—Significant differences between control and experimental groups ($p < 0.05$).

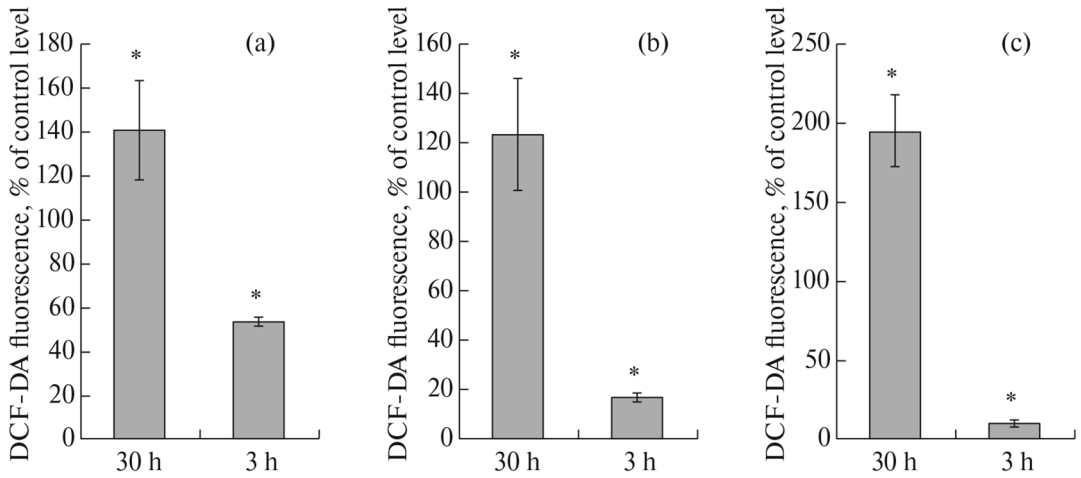

Fig. 4. Effect of oxygen deficiency on the ability of Pacific oyster *Crassostrea gigas* hemocytes to spontaneously generate reactive oxygen species (ROS). (a) Agranulocytes. (b) Hyalinocytes. (c) Granulocytes. The mollusks were kept for 24 h under conditions of oxygen deficiency: 30 h—experimental group, 30% oxygen saturation ($n = 10$); 3 h—experimental group, 3% oxygen saturation ($n = 10$). Spontaneous ROS production was by the fluorescence intensity of hemocytes stained with DCF-DA (final concentration in the sample, 10 μmol/L). Dye fluorescence was analyzed in the flow cytometer fluorescence channel 1 (FL1; 530/30 nm band pass). DCF-DA fluorescence intensity level is presented on the graph as % of the control level. *—Significant differences between control and experimental groups ($p < 0.05$).

Incubation of oysters under conditions of 30% oxygen saturation of water led to an increase in the spontaneous ROS production: in agranulocytes, on average, by 40% (Fig. 4a) and in granulocytes by more than 90%. At the same time, moderate hypoxic load had no significant effect on ROS production by hyalinocytes. Incubation in 3% oxygen-saturated water inhibited ROS production in all cell types (Fig. 4).

DISCUSSION

It is well known that, in bivalve mollusks, oxygen deficiency often causes a decline in the total count of circulating hemocytes and changes in the ratio of their types [23, 25, 28]. In our study, a deeper hypoxic load (3% of the normoxic level) led to a reduction in the percentage of granulocytes and hyalinocytes, as well as an increase in

the proportion of agranulocytes in oyster hemolymph. The opposite effect, an increase in the percentage of granulocytes, was detected in the group of oysters after incubation at 30% oxygen saturation of water.

Among the possible reasons underlying changes in the cellular composition of the oyster hemolymph, the following are most significant:

1. Proliferative activity of hemocytes and their precursors [25];
2. Death of a certain type of hemocytes [23];
3. Functional changeovers of one type of hemocytes into another (granulation vs. degranulation) [36, 37];
4. Migration of hemocytes into tissues [38].

Presumably, agranular cells represent immature hemocytes, the proliferation of which can occur directly in the hemolymph [39, 40, 41, 42]. Within a short-term experiment, it is unlikely that a change in the ratio of hemocyte types can be elicited by the proliferative activity in hematopoietic tissue. This is supported by the data on the absence of dividing hemocytes in oyster hemolymph samples in both experimental groups. Similarly, there were no changes in the proportion of dead cells in hemolymph during hypoxia. At the same time, granulocytes are characterized by degranulation and, as a consequence, an increase in the relative proportion of agranular cells in hemolymph [43]. The possible migration of granulocytes into tissues should also not be ruled out, since this type of cells is known to be able to actively relocate from the hemolymph vessels to the gills, mantle, and other tissues and organs of mollusks [44]. Thus, short-term changes in the cellular composition of hemolymph during hypoxia are implemented via rapid adaptive rearrangements, among which the most probable are degranulation of granulocytes and/or their migration into tissues under deep hypoxia, as well as the functional transition of agranulocytes into granulocytes at a moderate lack of oxygen. It is noteworthy that an increase in the proportion of granular cells at 30% oxygen saturation of water and its decrease after one-day exposure to deep hypoxia (3% of oxygen saturation of water of the normoxic level) may reflect the development of a compensatory adaptive response in *C. gigas* under conditions of moderate hypoxic load. The latter assumption is further confirmed by an increase in the level of DCF-DA fluorescence in agranulocytes and granulocytes after incubation of oysters at 30% oxygen saturation and its reduction in all cell types after 24-h hypoxia at 3% oxygen saturation of water. The changes we revealed in the level of spontaneous ROS production are generally consistent with the literature data. For example, in *M. coruscus* and *C. farreri*, oxygen deficiency induced an increase in ROS production [25], while the opposite effect was observed in *P. viridis* [23, 24]. The mechanisms underlying the effect of oxygen deficiency on the ability of hemocytes to generate an oxidative burst remain a matter of debate. The decrease in ROS production is thought to be due to a metabolic adjustment with the involvement of the HIF factor in response to hypoxic exposure [45]. It is also likely that the increase we detected in the ROS concentration in oyster hemocytes is a consequence of the reorganization of the mitochondrial respiratory chain, since mitochondria represent the main source of ROS in *C. gigas* hemocytes, and the lack of oxygen can induce changes in the mitochondrial electron transport chain [46, 47]. This assumption is indirectly confirmed by the data that the intracellular level of ROS in oyster hemolymph cells correlates with changes in the mitochondrial membrane potential under hypoxia [14].

Thus, the results of this work indicate that the pattern of changes in oyster hemolymph parameters is determined not only by the duration of hypoxic exposure, but also by the concentration of dissolved oxygen. The increase in the relative granulocyte count and the ability to generate an oxidative burst probably indicate the development of a compensatory response in the Pacific oyster due to exposure to a moderate lack of oxygen. Apparently, short-term incubation under conditions of 30% dissolve oxygen saturation occurs within the range of *C. gigas* tolerance to hypoxia. Deep hypoxia, in turn, induced a decrease in the percentage of granulocytes in hemolymph and a suppression of ROS production in hemocytes, exerting a suppressive effect on the cellular immune response in oysters.

AUTHORS' CONTRIBUTION

Conducting experiments and data analysis

(E.S.K., A.Yu.A., T.A.K.); graphic data representation and statistical data processing (E.S.K., T.A.K.); preparing and correcting a manuscript (A.Yu.K.).

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CONFLICT OF INTEREST

The authors declare that they have no evident or potential conflict of interest in relation with the publication of this article.

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