The influence of acute hypoxia on the functional and morphological state of the black scorpionfish red blood cells

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Abstract The investigation of the mechanisms of red blood cell steadiness to the oxygen lack in tolerant teleosts is of current scientific interest. Black scorpionfish, *Scorpaena porcus* L., is a widespread benthal species in the Black Sea and is highly resistant to hypoxic influence. The morphological state of black scorpionfish red blood cells under acute hypoxia was assessed using DNA-binding dye SYBR Green I and fluorescent microscopy. Changes in membrane potential of mitochondria and functional activity of cells were determined by rhodamine 123 (R123) and fluorescein diacetate (FDA) fluorescence. Oxygen deficiency leads to bidirectional changes in volume of erythrocytes and their nuclei. Between 0.57 and 1.76 mg O_2 l^{-1}, both parameters increased on 3–12 and 7–21%, respectively. At 1.76–4.03, cells shrank on 1.5–6.0% and nucleus size decreased on 1.5–3%. Acute hypoxia induced a significant increase of R123 (12–60%) and FDA (30–184%) fluorescence. These reactions are caused by a probable decrease in erythrocyte membrane permeability.

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Introduction

Hypoxia is a widespread phenomenon throughout the world's aquatic systems. That is determined by the low solubility of oxygen present in water (Levin 2002; Middelburg and Levin 2009) and progressive anthropogenic pressure on coastal waters (Diaz and Rosenberg 2008). Stable and significantly sizable areas with extremely low amounts of dissolved oxygen (less than 1.5 mg O_2 l^{-1}) occur in the Atlantic, Pacific, and Indian oceans (McEnroe et al. 1998; Joyce 2000; Levin 2002; Middelburg and Levin 2009). Global warming is considered to facilitate spreading of coastal hypoxic systems and leads to the qualitative transformation of the present ecosystems, increasing the role of organisms tolerant to oxygen deficiency (Gewin 2010).

Organisms which inhabit areas with low oxygen content for long periods of time are of particular interest (McEnroe et al. 1998; Joyce 2000; Gewin 2010). A hypoxic state is a functional norm for such species, and this fact assumes a significant reorganization of metabolic processes which are directed towards the optimization of an organism's energetic costs (Hochachka 1986).

The reaction of cell systems to hypoxia is of current interest in research. Nucleated red blood cells (RBCs) are one of the most frequently used cell types. Fish erythrocytes possess mitochondria, and Krebs cycle enzymes exist in normal somatic cells (Phillips et al. 2000). It has been shown that teleost erythrocytes are able to depress membrane and metabolic functions in conditions with low levels of oxygen in order to balance the depressed membrane and metabolic functions (Soldatov et al. 2014) and to simultaneously activate substrate phosphorylation (Jibb and Richards 2008). During this process, the appropriate morphological changes in red blood cells can be observed (swelling) (Cossins and Gibson 1997).

In vitro experiments have demonstrated that red blood cells' reactions under hypoxia play the key role in changes of hemoglobin oxygen-binding properties. The response of teleost RBC to hypoxia is controlled by blood catecholamines (Nikinmaa 2001). The decrease in oxygen partial pressure enhances the sensibility of erythrocytes' cell membrane to these compounds (Perry and Reid 1992; Lecklin et al. 2000). Blood catecholamines simultaneously increase the cellular aerobic metabolism and activate Na^+-H^+ exchanger (Motais et al. 1992). The last one causes immediate swelling of red blood cells (Hoffmann et al. 2009). The physiological meaning of erythrocytes' enlargement under hypoxia is the increase of hemoglobin oxygen affinity through (a) the raise of intracellular pH level (Motais et al. 1992; Bogdanova and Berenbrink 2009) and (b) the decline in organic phosphate level (Jensen and Weber 1989).

Besides fish red blood cells' swelling under hypoxic conditions is well studied, all experimental protocols include the addition of catecholamines into an incubation medium to stimulate the activity of Na^+-H^+ exchanger. So, it is hard to distinguish the direct influence of oxygen lack on red blood cells. It is unknown whether fish erythrocytes possess the ability to change their volume under hypoxia without influence of catecholamines. Such autonomic changes in volume were observed in mollusks' hemocytes (Novitskaya and Soldatov 2011).

The development of flow cytometric techniques allows an integral characteristic of the functional state of cell populations to be assessed using native cellular systems. In evaluation of hypoxia influence on red blood cells, it is important to consider the energy status of the cells and the state of their nuclei, mitochondria, and biomembranes. In this regard, suitable fluorochromes are rhodamine 123 (R123), SYBR Green I, and fluorescein diacetate (FDA).

R123 is one of the most widespread fluorochromes used in the analysis of the mitochondria. The lipophilic nature of the dye allows it to pass easily through polarized membranes, to accumulate specifically in mitochondria, and not to color other cell membranes (Shapiro 2000; Toescu and Verkhratsky 2000).

The vital dye, FDA, is used to assess the general metabolic activity of cells (Diaper et al. 1992; Jochem 1999; Stauber et al. 2002). Similar to R123, this fluorochrome is lipophilic and it passively crosses the cytoplasmic membrane. The transition of dye to the fluorescent state is carried out by nonspecific cellular esterases (Jochem 1999). It is also known that the intracellular turnover of FDA may be used to determine a membrane's integrity (Prosperi et al. 1985). The speed of dye release from the cells after enzymatic hydrolysis is lower than the speed of its entrance, which makes the decrease in FDA luminosity, an indicator of the damages in membrane (Prosperi et al. 1985).

SYBR Green I is frequently used for detection of nucleic acids in PCR analysis, fluorescence imaging techniques, and flow cytometry in various fields of biology and medicine (Marie et al. 1997; Zipper et al. 2004).

The aim of this paper is to investigate the functional and morphological states of *Scorpaena porcus* red blood cell suspensions under dosed hypoxia (in vitro) using flow cytometry and fluorescent microscopy methods.

Materials and Methods

Capture and transportation of the material The object of the research was benthal species *S. porcus* L. (black scorpionfish), which is widespread throughout the Black Sea. Black scorpionfish are highly tolerant to hypoxia and can survive in water with saturated oxygen levels of 15–20% without symptoms of asphyxia for long periods of time (Shulman et al. 2003). Fifteen adult specimens were chosen for the experiment (body length 10.7–17.0 cm, weight 83.6–133.7 g). Before starting the experiment, animals were kept in 50-l aquariums equipped with a flowing seawater system for an acclimation period of roughly 3 wk. There were five individuals in each aquarium (10 L of water per scorpion fish), temperature of 18–22°C. The concentration of oxygen in the water was maintained at a level of 7.5–8.0 mg O_2 l^{-1}.

Preparation of erythrocyte suspension Blood was obtained from the caudal artery. Heparin (Richter, Hungary) was used as anticoagulant. Erythrocytes were separated from the plasma by centrifugation (1000g for 15 min) (centrifuge MPW-310, Poland) and washed three times in an equivalent volume of the following solution: 128 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 15 mM Tris, and 2.2 mM D-glucose (pH 7.8) (Tiihonen and Nikinmaa 1991). The incubation saline was sterilized beforehand at a temperature of 95°C for a period of 3 h. After removal of the supernatant, the residue of erythrocytes was used in experiments in vitro.

Hypoxia modeling Hypoxic conditions were created by bubbling the incubation medium with nitrogen gas for a period of 15–120 s. The oxygen concentration range used in the experiment was 0.57–8.17 mg O_2 l^{-1}. The level of oxygen was measured using a polarographic membrane oxygen sensor.

Erythrocytes amounting to 0.15 ml were placed in VACUETTE™ (6.0-ml) plastic vacuum tubes, which then were filled with the incubation saline solution with the corresponding level of oxygen. Cell concentration in suspension was 4.5×10^7 cells ml^{-1}. The control tube was prepared similarly: the oxygen content was preserved at the level of 7.5–8.0 mg O_2 l^{-1}. The hypoxic exposure of red blood cells lasted for 4 h at a temperature of 18–20°C. Sampling tubes were connected to a special system (Fig. 1).
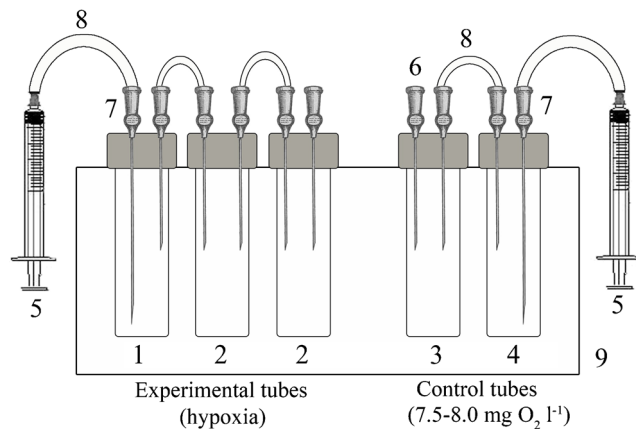
Figure 1. Experimental stand. (*1*) Vacuum tube with test sample. (*2*) Vacuum tube with incubation medium with given oxygen concentration. (*3*) Vacuum tube with the incubation medium fully saturated with oxygen (control). (*4*) Vacuum tube with control sample. (*5*) Insulating all-glass 1-ml syringe. (*6*) Syringe needle (thickness 0.7 mm). (*7*) Needle for sampling suspensions for flow cytometric analysis. (*8*) PVC pipe (diameter 5 mm). (*9*) Support.

The test tube (1) was connected with two tubes containing the incubation medium of the given oxygen concentration (2); for the control group, only one tube with the incubation medium (3) was used. This system of tubes was linked with syringes (0.7 mm) (6) and PVC pipes (5 mm) (8) and prevented the appearance of air bubbles in the test and control samples. When the material was sampled from the bottom of the tube, the erythrocyte suspension was diluted automatically with the incubation medium of the given oxygen concentration. Two spare test tubes with the incubation saline in the test sample provided a more accurate observance of hypoxic conditions within the vacuum tube with red blood cells. Thus, the effect of reoxygenation was excluded.

Dyeing protocol After exposition to hypoxia, the red blood cells were dyed with the vital fluorochromes: FDA, SYBR Green I, and R123 (Molecular Probes, Eugene, OR). Equal volumes of suspension were obtained from the control and test tubes and diluted 100 times in the incubation solution of the given oxygen concentration. The cell suspensions were incubated simultaneously for 30 min with FDA and for 10 min with R123 at room temperature. The final concentration of R123 in the solution was 2.5 μl ml^{-1}, and that of FDA was 10 μl ml^{-1}.

SYBR Green I was used for DNA analysis in red blood cells' nuclei. The final dilution of the dye in the cell suspension was 10^{-4}. Drying was carried out 20 min.

Flow cytometric measurements were done on a Cytomics FC500 cytometer (Beckman Coulter, Brea, CA).

Morphometry of red blood cells Simultaneously with the cytometric measurements, microphotography of stained by SYBR Green I red blood cells was carried out. The cells were photographed on an inverted laboratory fluorescent microscope (Nikon Eclipse TS100 equipped with camera (Ikegami ICD-848P), in both optical and fluorescent modes, with a set of filters for agitation of the blue region of the spectrum for SYBR Green I). Pictures of erythrocytes in a thick drop were taken after the cells' sedimentation on the surface of the microscope slide. Thus, for each group of cells, two photos were obtained: an illuminated microscope field with discrete cell contours and a darkened one where fluorescent nuclei were visualized. Further image processing was carried out in Adobe® Photoshop® (CS3 Extended version 10.0) by overlaying the dark images onto the light images and setting the transparency of the upper layer to 20–40%. Pictures obtained were used for further analysis (Andreyeva and Mukhanov 2012).

Subsequent measurements of morphometric parameters of nucleated red blood cells were done by using the ImageJ 1.44p (Girish and Vijayalakshmi 2004). The following indexes were measured: cell length (C_1), cell width (C_2), nucleus length (N_1), and nucleus width (N_2) (Fig. 2).

The cell volume (V_c) was calculated using the Houchin equation (Houchin et al. 1958)

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

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

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

Data analysis Flow cytometry data was analyzed in the program Flowing Software 2.0. The intensity of fluorescence was assessed on two-parameter histograms (channel FL1 in the green spectral region, 525 nm). The number of measurements for morphometric analysis was 100 cells per probe. The normality of distribution was verified using Pearson's criteria. The authenticity of differences was assessed with Student's test ($p \leq 0.05$, $p \leq 0.02$). Graphic representation of data was made in the program Grapher 7.0. Results are presented as $M \pm m$.

Results

Morphometric characteristics of black scorpionfish red blood cells Figure 3*a* illustrates changes in cell volume of red blood cells under hypoxia. It appears that for moderate concentrations of oxygen (1.76–4.03 mg O$_2$ l^{-1}), the erythrocyte volume decreased by 1.5–6.0%. At 1.76 and 2.14 mg

Figure 2. Morphological monitoring of red blood cell. Red blood cell dimensions were cell length (C_1), cell width (C_2), nucleus length (N_1), and nucleus width (N_2) and were made manually in the ImageJ.
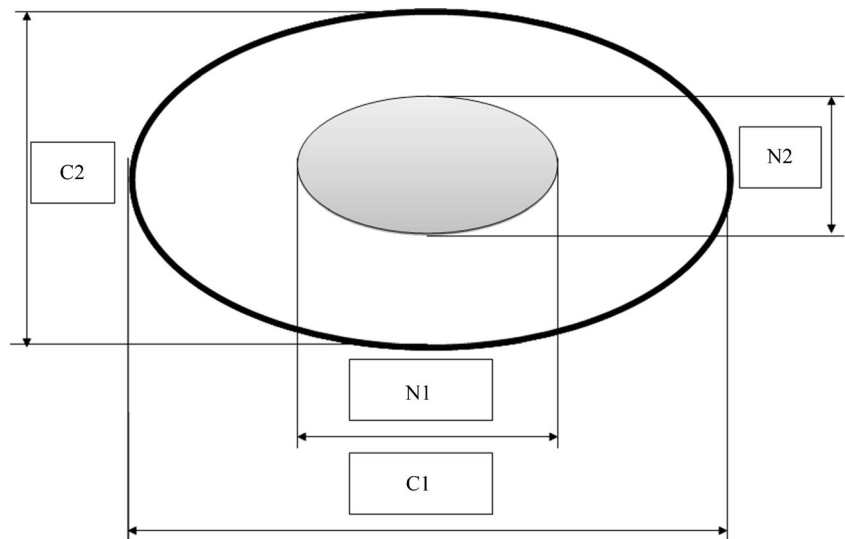
$O_2 \ l^{-1}$, the differences between the control and experimental groups were statistically significant. When the erythrocytes were suspended in a saline solution containing extremely low oxygen levels (0.57–1.76 mg $O_2 \ l^{-1}$), we observed the opposite reaction: the volume of red blood cells increased by 3–12%. For four experiments (1.07, 1.23, 1.50, and 1.73 mg $O_2 \ l^{-1}$), the changes were statistically significant (*$p < 0.05$; **$p < 0.02$).

Figure 3b indicates that under hypoxia (in vitro), the nucleus volume of red blood cells changed in proportion to the cell volume. At higher levels of oxygen (1.76–4.03 mg $O_2 \ l^{-1}$), during two points of experiment (2.14 and 4.03 mg $O_2 \ l^{-1}$), we observed a decrease in this parameter of 1.5–3%. Only one case of nucleus enlargement occurred for this level of hypoxia: at 3.58 mg $O_2 \ l^{-1}$, the volume of the organelle increased by 8%. At lower oxygen concentrations in the incubation medium (0.57–1.76 mg $O_2 \ l^{-1}$), the changes of parameter were less ambiguous. At five of eight points in the experiment, the nucleus volume increased by 7–21%.

The relationship between changes in erythrocytes and their nucleus volumes was confirmed by correlation analysis (Fig. 4a). The value of the R^2 coefficient was 0.71. No significant changes in the nucleocytoplasmic ratio between the control and the experimental groups were observed (data not shown). This fact also confirms the occurrence of this type of relationship between the size of cells and that of their nuclei.

Flow cytometric analysis of black scorpionfish red blood cells Hypoxia caused a significant increase in SYBR Green I fluorescence, especially at extremely low oxygen concentrations (0.57–1.76 mg $O_2 \ l^{-1}$). At the same time, a strong relationship (R^2 −0.81) between changes in nucleus volume and SYBR Green I fluorescence was observed (Fig. 4b).

Under hypoxic conditions, the increase in intensity of R123 fluorescence was observed in the erythrocyte suspension. The CV (coefficient of variation) of histogram experienced no noticeable changes—according to this criterion, the homogeneity

Figure 3. Changes in the volume of cells and their nuclei in hypoxic conditions (in vitro). (a) Changes in cell volume. (b) Changes in nucleus volume (*$p < 0.05$; **$p < 0.02$).
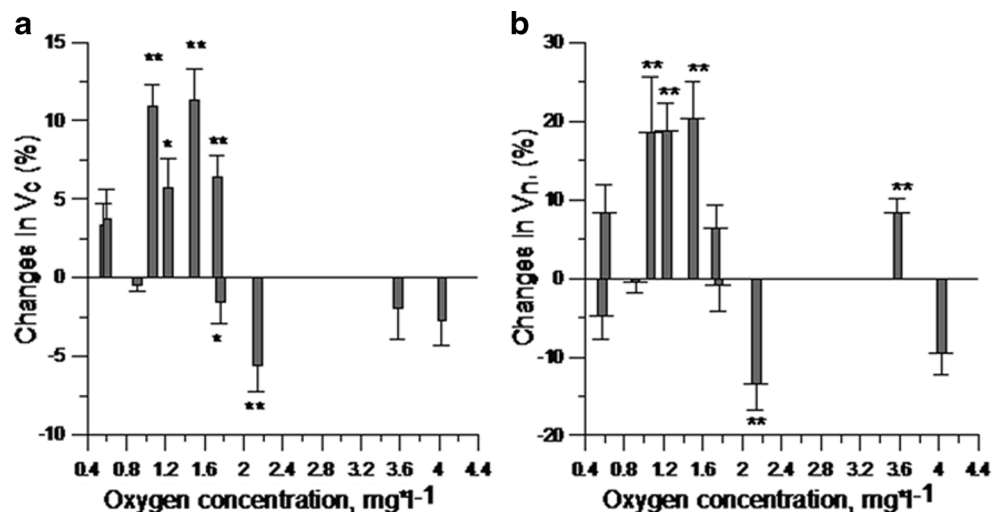
Figure 4. Comparison of the changes in the nucleus volume and changes in cell volume and SYBR Green I fluorescence. (*a*) Relationship between the cell size and nucleus size. (*b*) Relationship between the nucleus volume and SYBR Green I fluorescence.
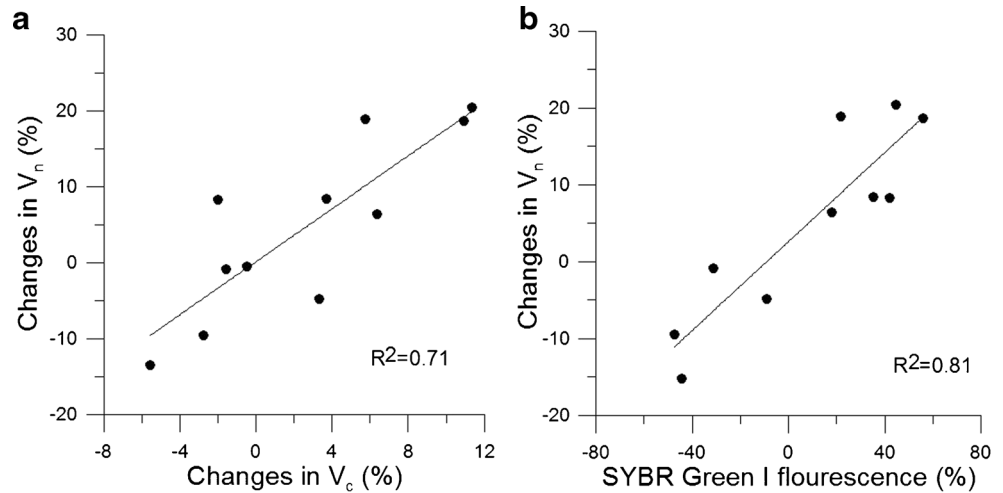
of distribution of the red blood cell populations was maintained at the same level.

The values of intensity in R123 fluorescence in relation to the control level are shown in Fig. 5*a*. At five points of the experiment (0.91, 1.03, 1.39, 1.76, and 3.44 mg O_2 l^{-1}), changes were statistically significant and amounted to 8–61% ($p \leq 0.05$). In the remaining variations of hypoxia, we only observed a tendency for increase not exceeding 18%. As demonstrated in Fig. 5*a*, R123 fluorescence did not depend on the degree of hypoxic influence. It appears that it may be linked to the experimental procedure: for each individual experiment, a new scorpion fish has been used.

Figure 5*b* shows that hypoxia led to a significant increase of FDA fluorescence in red blood cells. In nine out of ten experimental variations, the average values increased to 27–184% ($p \leq 0.02$). We did not observe any correlation between the fluorescence of FDA and the oxygen concentration in the incubation medium which is similar to R123. The reasons for this fact are mentioned above.

Despite the same trend of changes in the R123 and FDA fluorescence in red blood cells coincided, no correlation between these processes was observed (Fig. 6). The value of the determination coefficient (R^2) did not exceed 0.15. This fact means that the fluorescence of R123 and FDA reflects independent processes, which occur in red blood cells in conditions arising from a lack of oxygen.

Discussion

Information available on the fish red blood cells' shrinkage in hypoxic conditions is extremely limited. The same reaction of black scorpionfish erythrocytes was observed under short-term hypoxia (90 min, in vivo experiment) (Soldatov et al. 1994). It is known that the regulatory volume decrease in red blood cells may occur due to the activity of the K^+/Cl^- cotransporter and also by the movement of organic osmolytes (Jensen 1995). Among the possible ways for the channel

Figure 5. The intensity of R123 (*a*) and FDA (*b*) fluorescence in red blood cell suspensions under hypoxic conditions (in vitro, *$p \leq 0.05$).
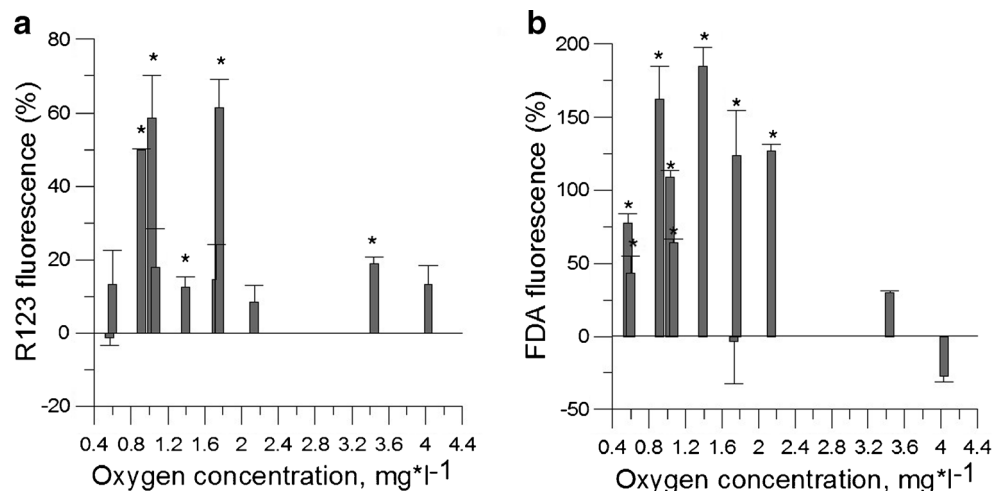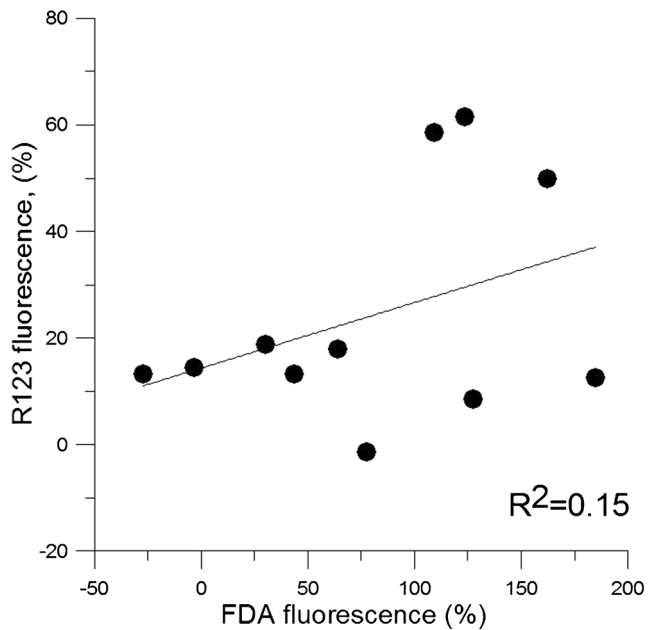
Figure 6. Relationship between the fluorescence of R123 and FDA in scorpion fish red blood cells in hypoxic conditions (in vitro). The *line* shows the weak correlation between the fluorescence of R123 and FDA and the independence of processes which underlie dye binding.

activation, the most probable one is a slight decrease of pH in the intracellular medium (up to 7.0) which may be the consequence of anaerobic processes and the accumulation of lactate in the cytoplasm (Adragna et al. 2004). Recent data suggest that nucleated red blood cells of lower vertebrates are able to switch their metabolism to anaerobic pathways in unfavorable conditions (Walsh et al. 1990).

Red blood cell swelling is a widespread reaction in hypoxic conditions; it has been well described in many studies (Jensen and Weber 1989). The enlargement of erythrocytes in hypoxic conditions appears due to the entry of Na^+ ions through the Na^+/H^+ antiport. The most relevant stimuli to the pathway activation are catecholamines which are released into the blood when the concentration of oxygen decreases. These agents interact with the β-adrenergic receptors of erythrocytes (Borgese et al. 1987). But during the in vitro experiment, such processes are excluded. Motais et al. (1992) has shown that Na^+/H^+ channel activity also increases when significant cytoplasm acidification occurs, as the affinity of inner cell's membrane to H^+ ions increases. In particular, significant intracellular cells acidosis inhibits K^+/Cl^- cotransporter that also assists cytoplasm hydration in red blood cells (Adragna et al. 2004). Under extreme hypoxia (0.57–1.76 mg O_2 l^{-1}), this state is quite acceptable and describes the changes in cell volume noted at our work. Thus, different changes in cell volume under hypoxia (cell enlargement between 0.57 and 1.76 mg O_2 l^{-1} and cell shrinkage at 1.76–4.03 mg O_2 l^{-1}) are explained by a gradual decrease in erythrocyte pH. This is the most probable mechanism of hypoxia influence on cell volume in vitro. During in vivo experiments, the activation of the K^+/Cl^- channel exists unlikely because of quick release of catecholamines into the blood and their interaction with β-adrenergic receptors within the cell membrane.

We have previously shown that hypoxia caused changes in nucleus volume of black scorpionfish red blood cells. At oxygen concentration in the incubation medium of 0.57–1.76 mg O_2 l^{-1}, the organelle size increased by 21% in some experiments ($p < 0.02$). Similar effects of hypoxia have previously been shown during in vivo experiments (Soldatov 1996). Nuclei enlargement usually occurs in the following cases (Tashke 1980): polyploidy, hydration and the functional activation of the structure (the increase in euchromatin content), and toxic influence.

It is clear that the proliferative activity of mature red blood cells is suppressed. It is lost at the stage of basophilic normoblast (Soldatov 1996). Cytokines (amitosis) induced by hypoxia are possible (Soldatov 1996), but it is not accompanied with DNA reduplication and therefore does not lead to changes in SYBR Green I fluorescence. Any toxic effect on the red blood cells during our experiments is also excluded.

The hydration of nucleus matrix as a process which describes the increase in the structure's volume is also possible. The relationship between changes in red blood cell volume and its nuclei supports this interpretation. The absence of differences in nucleocytoplasmic ratio in control and experimental probes also confirms the hypothesis of hydration.

However, changes in nucleus volume do not appear to be the linear function of changes in cell size (Lay and Baldwin 1999). A strong relationship between SYBR Green I and nucleus size should also be taken into account (R^2 −0.81). This allows assuming the occurrence of nucleus reactivation under extremely low oxygen content in the incubation medium, because changes in nucleus size depend on the level of chromatin condensation (Hyde 1982). At the same time, the interpretation of the increase in SYBR Green I fluorescence has some difficulties; thereupon, the mechanism of the dye interaction with DNA is not clear yet (Zipper et al. 2004). The available information does allow us to conclude, however, that the intensification of SYBR Green I fluorescence is practically always coupled with an increase in functional cell activity (Cerca et al. 2011).

It has been shown previously that the accumulation of R123 in cells is a result of internal molecular interactions between the dye and proteins in the membranes of the mitochondria (Weinberg et al. 2000). The displacement and distribution of fluorochrome occurs in compliance with the Nernst potential (Toescu and Verkhratsky 2000). This fact explains the dependence of R123 fluorescence on the value of mitochondrial membrane polarization. The majority of research has shown that hypoxia restricts the accumulation of R123 in cells. This has been demonstrated in the studies carried out on neurons and smooth human muscle fibers that are

sensitive to oxygen deficiency (Bahar et al. 2000; Perez Velazquez et al. 2000; Wang et al. 2005). The decrease in intensity of fluorescence is caused by reduction in mitochondrial membrane potential due to the inactivation of the respiratory chain (Weinberg et al. 2000). The increase in R123 fluorescence under hypoxia observed during our experiments has not been previously described in other studies. This may be explained by the fact that scorpion fish is extremely tolerant to oxygen deficiency. Its critical oxygen level and lethal oxygen level are one of the lowest values among other representatives of Black Sea fish fauna: 1.17 and 0.38 mg O_2 l^{-1}, respectively (Soldatov 2011). The most likely reason for the increase of R123 fluorescence in erythrocyte suspensions is the reduction of cell membrane permeability. Hochachka (1986) described this phenomenon as "metabolic arrest": the limitation in the number of functionally active ionic channels by their conformational reorganization. This allows a reduction in energy costs for the maintenance of transmembrane ion gradients and therefore lowers functional pressure on mitochondria. Similar reactions of cell systems have been observed in organisms which are tolerant to extreme environments (hypoxia, hypothermia) (Hochachka 1986). We have previously shown that black scorpionfish red blood cells are able to maintain a balanced depression of membrane and metabolic functions in conditions of oxygen deficiency (Soldatov et al. 2014). This fact probably explains the increase in R123 fluorescence in erythrocyte suspensions in a hypoxic environment. The increase in R123 fluorescence may be also a consequence of the initial phase of red blood cells' reactions to hypoxia, notably the synthesis of hypoxia-inducible factors (HIFs) and the switching of the cell's metabolism to the anaerobic pathway. This hypothesis is quite possible since it is well known that the expression of HIF-1α begins in few hours after the oxygen concentration in environment decreased (Rus et al. 2011). This period of time corresponds to the duration of our experiment (4 h).

As discussed previously, hypoxia substantially influenced FDA fluorescence in red blood cells: it increased significantly. The increase in the intensity may be determined by the next list of processes:

- Increase in activity of cell esterases
- An alkalization of intracellular medium
- Changes in cell membrane permeability

Since the transformation of dye in a luminous form is carried out by esterases (Prosperi 1990), the intensity of FDA fluorescence may indicate changes in the activity of lipid metabolism enzymes. For this reason, the increase in dye fluorescence is frequently explained as intensification of cell metabolism (Heslop-Harrison and Heslop-Harrison 1970). This kind of interpretation may be used to explain the observed changes. However, the absence of correlation between R123

and FDA fluorescence does not allow us to definitively accept this point of view. It is also unlikely possible that the increase in pH level in cytoplasm played an important role in the changes of FDA fluorescence in red blood cell suspensions under experimental hypoxia. It is generally known that an intensification of anaerobic processes in cells leads to the accumulation of lactate and the appearance of local acidosis in cells (Baldisserotto et al. 2008). The most likely reason for the increase in FDA fluorescence in red blood cells is the abovementioned reduction in membrane permeability. It is also known that the polar fluorescent form of FDA is unable to cross the outer cell membranes quickly. When permeability decreases, fluorochrome accumulates in cells (Prosperi 1990).

Conclusions

Thus, under hypoxia, the volume of erythrocytes and their nuclei changed consecutively. During the initial stages of hypoxia (1.76–4.03 mg O_2 l^{-1}), both structures reduced in size, and subsequently between 0.57 and 1.76 mg O_2 l^{-1}, a significant increase was observed. At extremely low oxygen concentrations, SYBR Green I fluorescence in red blood cells' nuclei increased and the changes observed correlated with changes in volume of the organelle (R^2 −0.81). Experimental hypoxia in vitro induced an essential increase in R123 and FDA fluorescence intensity in black scorpionfish red blood cells. This reaction is based on a probable loss in the cell membrane's permeability. This process assists the accumulation of FDA polar fluorescent form in cells and simultaneously lowers energetic costs on the maintenance of transmembrane cationic gradients. The last one reflects an increase in mitochondrial membrane potential and is indicated by the intensification of R123 fluorescence in erythrocyte suspensions.

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