STUDY OF THE RELATIONSHIP BETWEEN THE FUNCTIONING OF THE PURINERGIC SIGNALING SYSTEM AND CHANGES IN THE COMPOSITION OF PROTEINS AND MORPHOFUNCTIONAL CHARACTERISTICS OF ERYTHROCYTES

N.V. Revina, N.V. Gromova[*]*, S.S. Bochkareva, E.S. Revina, T.P. Kuzmenko, V.I. Incina, V.V. Revin, M.V. Parchaykina*

Federal state-financed academic institution of higher education «National Research Ogarev Mordovia State University», 68 Bolshevistskaya St., Saransk, Republic of Mordovia, 430005, Russia.

* Corresponding author: nataly_grom@mail.ru

Abstract. Erythrocytes, performing their basic oxygen transport function, simultaneously affect blood viscosity by changing the deformability of their structure, and also have a vasodilating effect on the walls of blood vessels using NO. An important role in this regulation is played by the purinergic signaling system, which was confirmed by this study of the morphofunctional parameters of red blood cells in the presence of ATP and sodium nitroprusside (SNP). We found that under the action of 0.5 mM ATP on red blood cells, the ability of hemoglobin (Hb) to bind oxygen decreases against the background of a slight increase in complexes with oxygen, while the total number of membrane-cytoskeletal proteins also decreases. This, in turn, is accompanied by a redistribution of Hb molecules and an increase in the area of red blood cells. In the presence of 5.0 mM ATP, the oxygen transport function of erythrocytes and the quantitative composition of membrane proteins change similarly, while the geometric height and volume of the cells are significantly reduced. The combined effect of 100 μM SNP and 0.5 mm ATP has the greatest effect on the conformation of hemoporphyrin molecules, which leads to a sharp increase in hemoglobin complexes with oxygen, while the affinity of hemoglobin for oxygen decreases. The membrane-cytoplasmic component of the cell also undergoes changes. Thus, ATP, both separately and in combination with SNP, affects the oxygen transport and regulatory function of red blood cells, activating the purinergic signaling pathway and triggering a cascade of adaptation reactions in the cell.

Keywords: erythrocyte, hemoglobin, cytoskeleton, ATP, nitric oxide.

List of Abbreviations

GAPDH – glyceraldehyde-3-phosphate dehydrogenase
Hb – hemoglobin
LIM – laser interference microscopy
OPD – optical path difference
oxyHb – oxyhemoglobin
RBC – erythrocyte
SNP – sodium nitroprusside

Introduction

Recently, there have been findings that the spectrum of functions performed by red blood cells is much wider than the binding and transport of oxygen and metabolic gases derivatives (Ellsworth *et al*., 2009; Jensen, 2009).

It is known that red blood cells can autonomously regulate their own properties and functions adjusting to the specific condition and needs of the body. For instance, mammalian RBCs take part in hypoxic-induced reactions regulating blood flow (Grygorczyk & Orlov, 2017). They include two complementary mechanisms, the first of which is a rapid decrease in blood viscosity on account of increased deformability of RBC membranes.

The second mechanism is associated with a time-delayed and steady increase in the diameter of blood vessels due to the release of ATP and, accordingly, to the activation of purinergic receptors that stimulate NO and other vasorelaxants' production in vascular endothelial cells (Sidorenko *et al*., 2018). In this mechanism, ATP is a key factor regulating processes within cells as well as those related to the interaction of select body systems (Chaffey *et al*., 2003; Yawata, 2003).

First of all extracellular ATP and other nucleotides function through purinergic cell receptors (Burnstock & Knight, 2004).

Purinergic signaling activates surface P1 and P2 cell receptors by extracellular nucleosides and nucleotides such as adenosine and adenosine triphosphate, respectively. P2 receptors include P2X and P2Y – receptors and have well established roles in white blood cells and platelet biology. Recent findings points to the important role of these receptors in red blood cells. P2 receptor's activation stimu-

lates a number of signaling pathways in erythroid progenitor (erythrogonium), leading to the formation of reactive oxygen intermediates and apoptosis. In addition, P2 receptors' activation stimulates in mature RBCs signaling pathways mediating RBC volume regulation, eicosanoid release, phosphatidylserine effect, hemolysis, impaired ATP release and susceptibility or resistance to infection (Sluyter, 2015).

The RBC elastic properties are attributed to the interaction between the lipid bilayer and the cytoskeleton, which is a dynamic grid consisting mainly of spectran filaments linked by reconfigurable connective complexes (Bennett, 1990; Mohandas & Gallagher, 2008; Mohandas & Evans, 1994; Park *et al*., 2010).

Taking into account the above findings, it can be stated that red blood cells performing one of their main functions, namely, oxygen-transport, simultaneously bear on the blood viscosity due to their structural deformability, and also produce a vasodilating effect on the blood vessel walls by means of NO synthesis (Simmonds *et al*., 2014).

It can be assumed that red blood cells, blood plasma and vascular walls represent a single regulated system for the sustainable supply of certain tissues and organs with oxygen. And apparently, an important role in this regulation belongs to the purinergic signaling system.

Considering these findings, it was interesting to study the role of purinergic signaling system in the regulation of hemoglobin's oxygen-transport function, which was the main goal of our study.

The set goal implies a number of tasks: firstly, to explore the ATP effect as the main activator of RBC purinergic signaling system on morphofunctional and oxygen-transport functions; secondly to find the presence of changes in those parameters which bear on the RBC' s morphology and functional characteristics (phase state of the lipid bilayer and the protein composition responsible for the cytoskeleton formation, hematoporphyrin's conformational properties and hemoglobin's ligand-binding capacity). Thirdly, given the fact that the NO formation can be stimulated by activation of purinergic receptors, it is of interest to model the effect of a nitrogenous compound on hemoglobin morphofunctional characteristics and oxygen transport properties (Dietrich *et al*., 2000; Luneva *et al*., 2015).

Materials and Methods

Preparation of erythrocyte concentrate

Human peripheral blood RBCs obtained from the donors' whole blood at the Republican blood transfusion station served as the object of research. Donors were healthy men between 33-54 years old (n = 53; RBC $(10^{12}/l)_{mean}$ = 4.89 ± 0.08; Hb_{mean} = = 134.7 ± 4.69 g/l). All research stages were in compliance with the principles of World Medical Association Declaration of Helsinki (WMA Declaration of Helsinki). The research was also approved by the Local Ethics Committee of Mordovia State University (Minutes № 12 issued in September 17, 2014). Before the study, the subjects obtained written consent to participate in the studies. Sodium citrate (Sigma, USA) was used as an anticoagulant, the final concentration in the sample was 13 mM.

The blood was centrifuged to spin down formed elements (1000g 10 min). Plasma and leukocyte layer were discarded, the sediment was resuspended in a tenfold volume of erythrocyte washing medium (10 mM of KH_2PO_4, 3.5 mM of KCl, 1.5 mM of $MgCl_2$, 145 mM of NaCl, 6 mM of $C_6H_{12}O_6$, pH 7.4). The procedure was repeated three times, the resulting RBC's sediment was diluted in a ratio of 1:5.

RBC incubation

The resulting erythrocyte concentrate was thoroughly mixed, 10 ml was taken and placed in the incubation chamber with a constant temperature of +37 °C. The incubation time was 5, 15 and 30 minutes.

To study the effect of extracellular ATP on RBCs, a solution of hydrogenated ATP disodium salt ($C_{10}H_{14}N_5Na_2O_{13}P_3$ H_2O, M = 551.14 g/mol, Sigma, USA) was dispensed to the incubation medium with final concentrations of 0.5 mM, 1.0 mM and 5.0 mM.

To study the effect of nitric oxide donors, we used sodium nitroprusside ($C_5FeN_6Na_2O$, M = 262 g/mol, Sigma, USA,), which was added to purified erythrocyte concentrate sample with the final concentration of 100 μM).

Preparation of RBC membranes

Isolation of RBC membranes was carried out by means of J.T. Dodge method, 1963 (Dodge *et al*., 1963). The pre-purified RBC concentrate was lysed in a cooled to 0 °C 20-fold volume of the lyse solution (5 mM NaH_2PO_4; 0.5 mM PMSF, pH 8.0). The resulting mixture was incubated for 10 mins at t = = 4 °C, centrifuged (20000 g, 40 mins, t = 0 °C), the supernatant was discarded, the sediment was resuspended in the lyse solution. The procedure was repeated three times. The sediment was washed with a lyse buffer to obtain white membranes. Membranes were stored at t = – 60 °C.

Protein concentration measurement by Lowry method

The protein concentration in the obtained RBC membrane samples was measured by Lowry method using bovine serum albumin as the standard (Lowry *et al*., 1951). The optical path density was determined on a spectrophotometer (Shimadzu, Japan) at a wavelength of 650 nm.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis and Western blotting

The composition of RBC membrane and cytoskeletal proteins was studied by means of Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis using Laemmli method U.K.,1970 (Laemmli, 1970).

Electrophoresis was carried out on a BioRad apparatus using 4% stacking and 10% separating gels. Samples of RBC membranes were diluted with 5% β-mercaptoethanol buffer in a ratio of 1:2 and boiled for 5 minutes. The resulting mixture of 15 µl was planted to gel holes, electrophoresis was carried out at I = const (15 and 30 mA). The gels were stained with silver (Heukeshoven & Dernick, 1985). Calculation of the protein molecular weight is made against its electrophoretic mobility, using the method of regression analysis. Identification of RBC membrane proteins was verified as per Fairbanks–Steck classification (Fairbanks *et al*., 1971). Visualization, documentation and quantitative analysis of the obtained protein gels was made using gel documenting system Gel Doc XR+ (Alfred Nobel Drive, Hercules, California, USA). The results were processed in the ImageLab software.

To determine the specific proteins of erythrocytes (spectrin and band 3 protein) in the sample separated by polyarylamide gel electrophoresis, the Western blot method was used.

For confirmation, commercial test systems «SLC4A1 Antibody», SPTA1 Polyclonal Antibody (primary antibodies) and «Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488» (secondary antibodies) were used.

After electrophoresis, protein bands were transferred to PVDF membranes (Bio-Rad, USA), which were stained with Ponceau staining reagents.

Immunoblotting was performed according to the ECL Western Blotting protocols (Amersham Pharmacia Biotech, Sweden). The membrane was washed in PBSt buffer (PBS, 0.1% Tween-20) and nonspecific binding sites were blocked for 1 h at room temperature with 5% skimmed milk in the same buffer. At the end of the incubation, the membrane was washed in PBSt buffer and incubated with primary antibodies (rabbit polyclonal antibodies against SPTA1 and antibodies against SLC4A1 (dilution 1:1000 and 1:2000, respectively)) overnight at +4 ºC.

After incubation, the membranes were washed three times in PBS with 0.1% Tween-20 for 10 min. The membrane was then incubated for one hour with goat anti-rabbit Alexa Fluor Plus 488 (1:10,000 dilution). The membranes were then washed three times in PBS with 0.1% Tween-20 for 10 min. Protein bands were detected on a Gel-Doc XR+ gel-documenting system.

Laser interference microscopy

Structural changes in RBCs (RBC phase image area, RBC phase volume, RBC geometrical height) and hemoglobin redistribution (optical path difference (OPD), hemoglobin packing density m_{Hb}) were studied by laser interference microscopy (LIM) on an automated interference microscope MII-4M (LOMO, Russia) (Brazhe *et al*., 2008; Brazhe *et al*., 2006; Yusipovich *et al*., 2008; Minaev & Yusipovich, 2012; Yusipovich *et al*., 2009; Brazhe *et al*., 2009; Yusipovich *et al*., 2011; Mityanina *et al*., 2012). Interference images were processed using the FIJI software (FIJI, USA) (Schindelin *et al*., 2012).

Raman spectroscopy

The study of conformational properties of hemoglobin hemoporphyrin and determination of hemoglobin's ligand-binding capacity was carried out by means of Raman spectroscopy (Brazhe *et al*., 2018; Brazhe *et al*., 2014; Revin *et al*., 2016; Maksimov *et al*., 2001; Mikstacka *et al*., 2010; Rodnenkov *et al*., 2005). The analysis of the resultant spectra was made in OriginPro 2015 software (OriginLab, 2015).

Statistical analysis of results

The available findings were processed by means of variational statistics methodology on Microsoft Excel 2007 and STAT3 software package. The results are presented as arithmetic mean and standard deviation (Mean ± SD). Statistical processing of the experiment results was carried out by assessing the normality of values distribution for each of the samples using Geary criterion, homogeneity of dispersion, as well as ANOVA and ANOVA for repeated measurements. In case of statistically significant differences between the averages, we used Tukey method for comparing averages post-factum ($P \leq \leq 0.05$) (Tukey, 1949).

Results

Research into RBC morphofunctional characteristics exposed to different ATP concentrations and sodium nitroprusside / nitric oxide donor

To determine the RBC morphometric parameters, we used LIM, which allows to quantify the RBC optical properties, registering the OPD at each point of the cell, as well as RBC geometric dimensions. In addition, the RBC hemoglobin packing density was calculated m_{Hb}.

In donor samples, most of the RBCs were represented by normocytes, which OPD averaged 112.6 ± 7.6 nm, the RBC phase image area comprised 54.12 ± 2.1 μm^2, the physical height – 2.19 ± ± 0.14 mm, the RBC phase volume was 81.08 ± ± 3.45 μm^3 and hemoglobin packing density m_{Hb} – – 29.9 ± 1.6 pg (Fig. 1).

During incubation of RBCs with sodium nitroprusside RBC morphometric parameters were changing as follows: after 5 minutes of incubation OPD and RBC physical height decreased by 25.5% and 31.1%, respectively. At the same time, the remaining morphometric indicators increased (Fig. 2).

The increase in incubation time (30 minutes) led to a gradual increase in RBCs' OPD by 1.3 times compared to control samples. Simultaneously, there was an increase in the RBC phase image area, as well as the RBC phase volume and hemoglobin packing density by 14.9%, 48.15% and 19.8%, respectively.

At the same time, in addition to size, the shape of most erythrocytes also changed – outgrowths, characteristic of echinocytes, appeared (Fig. 3).

Exposure during incubation to different ATP concentrations also led to changes in the RBC morphometric parameters. The presence of 0.5 mM and 1.0 mM of ATP in the incubation medium caused the decrease in RBC OPD, physical height and phase volume. Simultaneously, the increase in RBC phase image area was detected, which was accompanied by low values of RBC hemoglobin packing density (Fig. 4, 5, 6).

The RBC exposure to increased ATP concentration (5.0 mM) by 30 minute of incubation led to an increase in OPD, RBC phase volume and hemoglobin packing density by 31.9%, 31.6% and 33.1%, respectively. The RBC phase image area did not change (Fig. 7, 8).

The exposure to different ATP concentrations and sodium nitroprusside simultaneously dispensed in the incubation medium also impacted the RBC morphometric parameters. Thus, the exposure to 0.5 mM of ATP and 100 μM of sodium nitroprusside led to the increase in RBC's OPD by 8% after 30 minutes of incubation. The RBC phase image area under these conditions did not significantly change, however, at the 15th minute of incubation, this indicator decreased by 21.8%. The RBC geometric height was decreasing throughout the incubation time. The RBC phase volume increased by 14.6% after 30 minutes of incubation. There was also a significant increase in the of hemoglobin packaging density at the 5th minute of incubation by 20.7% (Fig. 9).

When exposed to1.0 mM of ATP and 100 μM of sodium nitroprusside there was a simultaneous increase in RBC OPD and phase volume at the 15th and 30th minute of incubation (Fig. 10, 11). The RBC phase image area decreased at the 15th minute of incubation by 10%, by 30th minute the indicator reached the control level. The RBC physical height was less than the control level. Hemoglobin packing density statistically significant increased by 10.7% only by the 30th minute of incubation.

When incubated with 100 μM of sodium nitroprusside and 5.0 mM of ATP, an increase in RBC's OPD and phase volume by the 30th minute was observed by 44.6% and 23.5%, respectively. However, the RBC phase image area decreased, and the hemoglobin packing density increased by 20.9% by the 30th minute of incubation (Fig. 12, 13).

Therefore, we found that the RBC morphometric parameters, as well as the RBC hemoglobin packing density are being affected by ATP and sodium nitroprusside, whether they used separately or together.

Research into the quantitative composition of RBC membrane proteins and cytoskeleton exposed to ATP different concentrations and sodium nitroprusside / nitric oxide donor *

An important role in changing RBC's morphological characteristics is played by the functional state of their membranes. The bilipid component of the membrane is impacted by both extracellular substances (ATP, sodium nitroprusside) and intracellular functional state, causing changes in the composition of the membrane and cytoskeleton protein component. Therefore, in this set of experiments we have looked into changes in the qualitative and quantitative composition of RBC proteins.

The Lowry method revealed a change in the amount of total protein in RBCs exposed to sodium nitroprusside and different concentrations of ATP. A decrease in the total protein concentration was found, moreover, the most significant changes occurred when RBCs were exposed to sodium nitroprusside for 30 minutes: a decrease by 57.3%; as

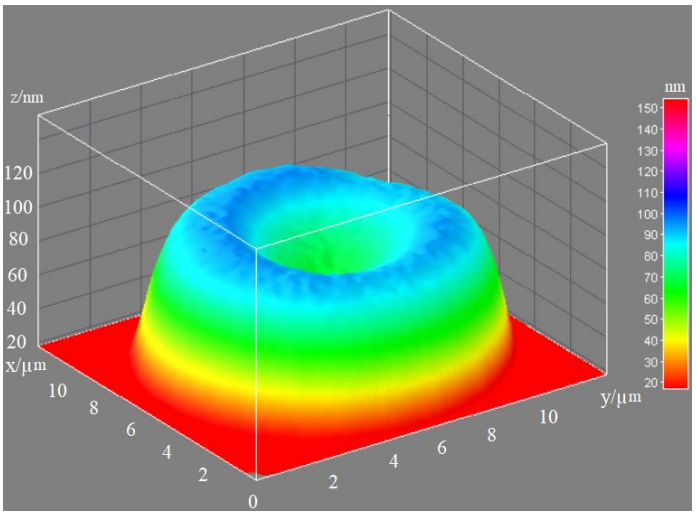

Fig. 1. Phase images of erythrocytes, taken using LIM (oX, oY: erythrocyte sizes, μm; oZ: optical path difference (OPD), nm): erythrocyte from a healthy donor (discocyte)

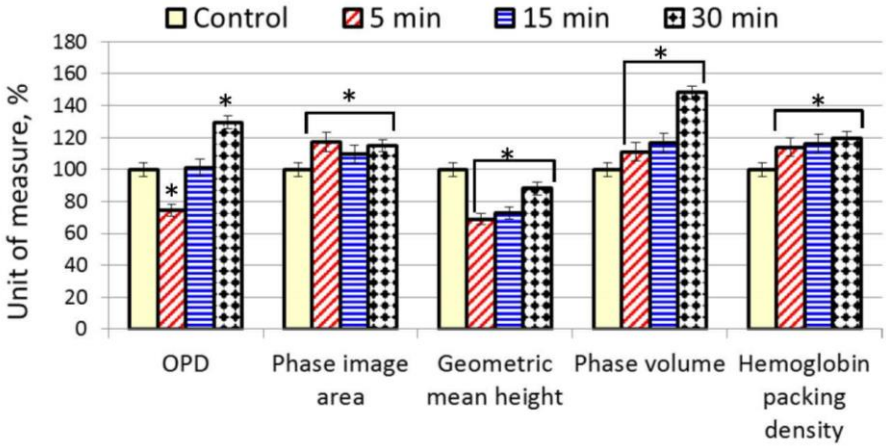

Fig. 2. RBC morphometric parameters after exposure to 100 µM of sodium nitroprusside (* – $P \leq 0.05$)

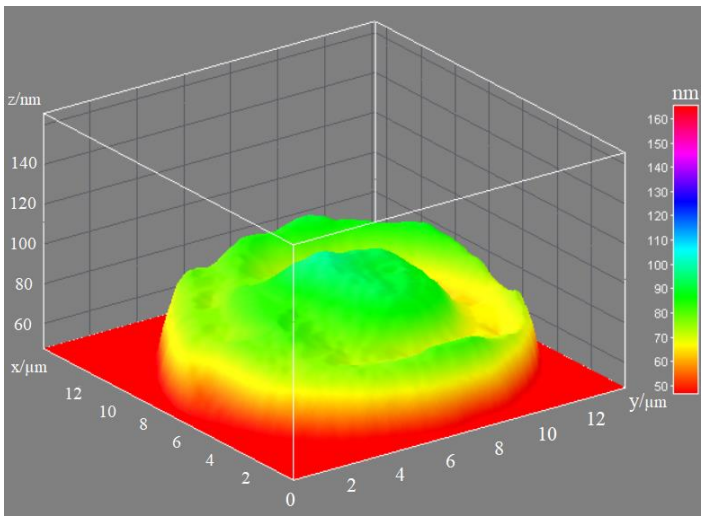

Fig. 3. Phase images of erythrocytes, taken using LIM (oX, oY: erythrocyte sizes, μm; oZ: optical path difference (OPD), nm): erythrocyte (echinocyte) after exposure to 100 µM of sodium nitroprusside (30 minutes)

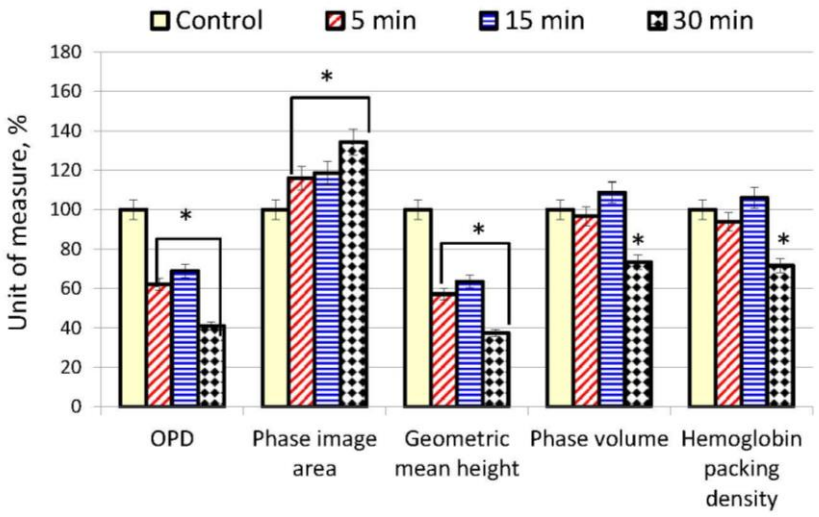

Fig. 4. RBC morphometric parameters effected by 0.5 mM of ATP (* – $P \leq 0.05$)

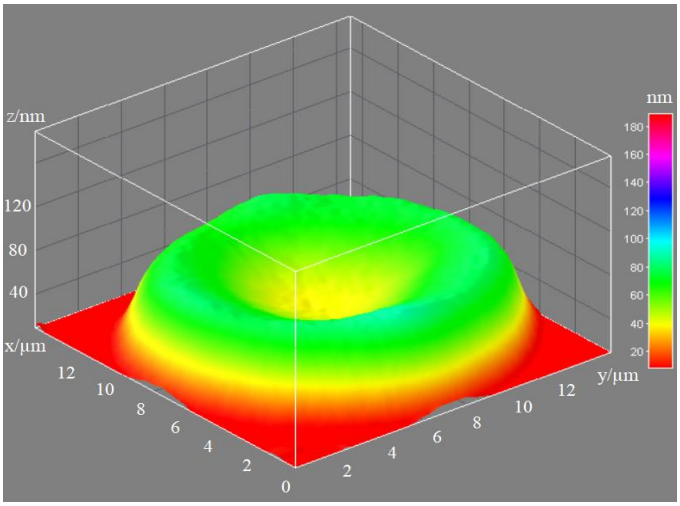

Fig. 5. Phase images of erythrocytes, taken using LIM (oX, oY: erythrocyte sizes, μm; oZ: optical path difference (OPD), nm): erythrocyte after exposure to 0.05 µM of ATP (30 minutes)

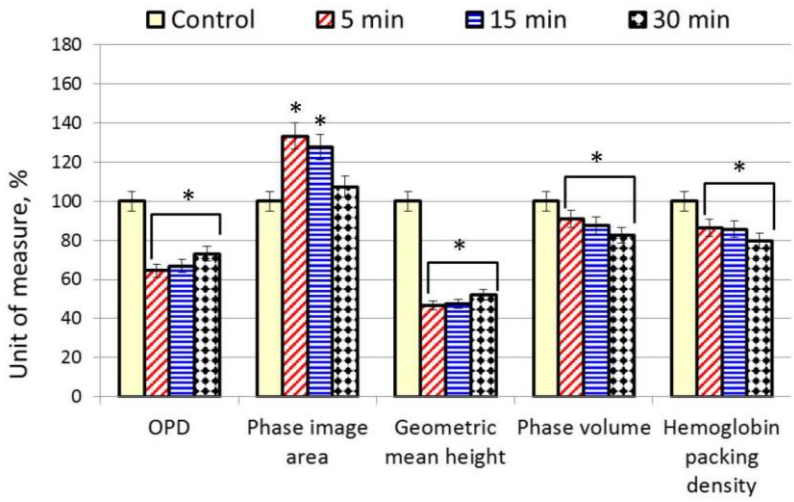

Fig. 6. RBC morphometric parameters effected by 1.0 mM of ATP (* – $P \leq 0.05$)

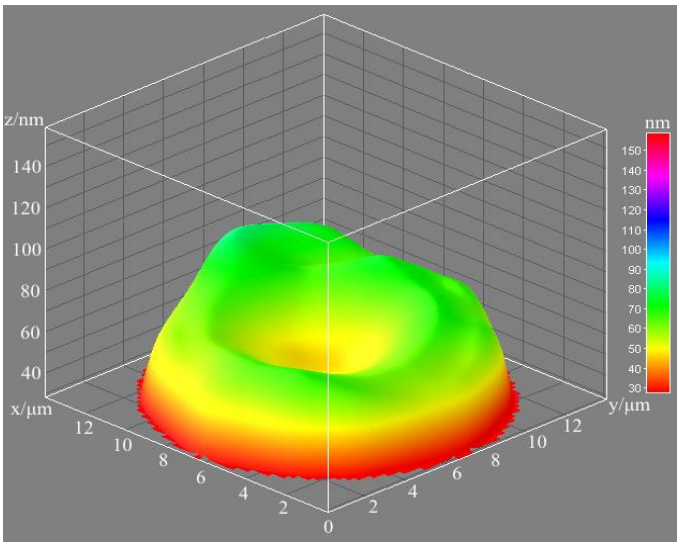

Fig. 7. Phase images of erythrocytes, taken using LIM (oX, oY: erythrocyte sizes, μm; oZ: optical path difference (OPD), nm): erythrocyte after exposure to 5.0 μM of ATP (30 minutes)

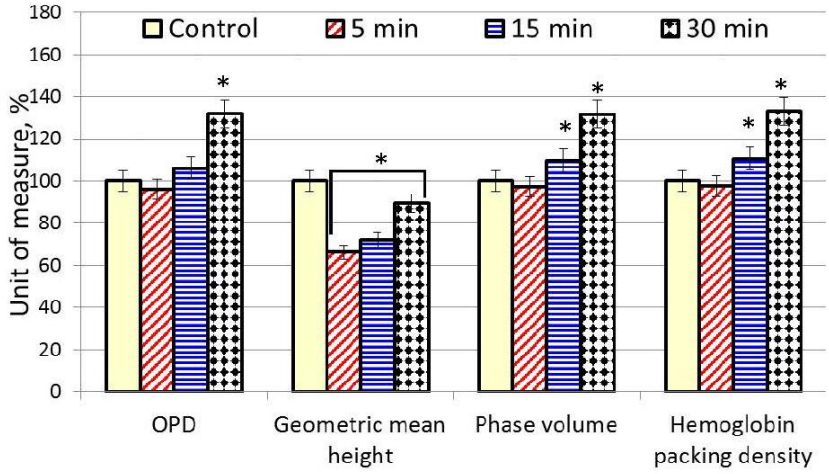

Fig. 8. RBC morphometric parameters effected by 5.0 мМ of ATP (* – $P \leq 0.05$)

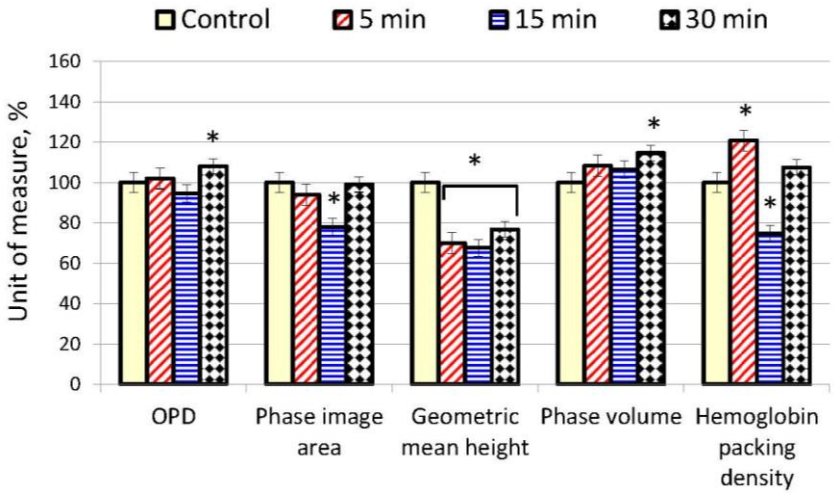

Fig. 9. RBC morphometric parameters after exposure to 0.5 mM of ATP and 100 μM of sodium nitroprusside (* – $P \leq 0.05$)

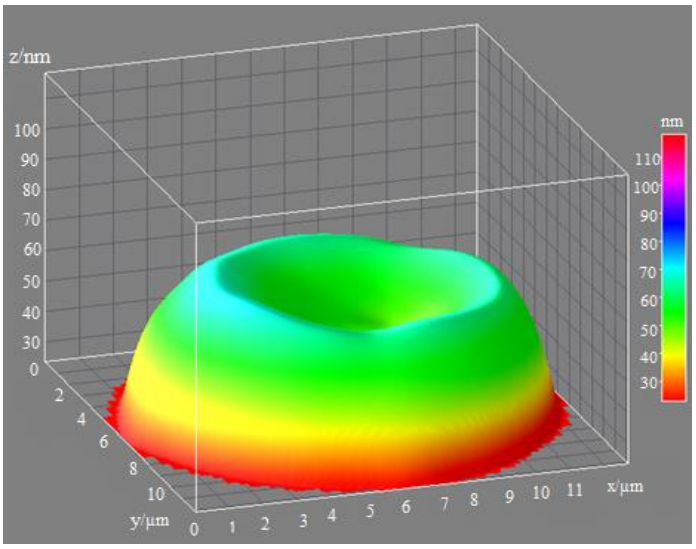

Fig. 10. Phase images of erythrocytes, taken using LIM (oX, oY: erythrocyte sizes, μm; oZ: optical path difference (OPD), nm): erythrocyte after exposure to 1.0 µM of ATP and 100 µM of sodium nitroprusside (15 minutes)

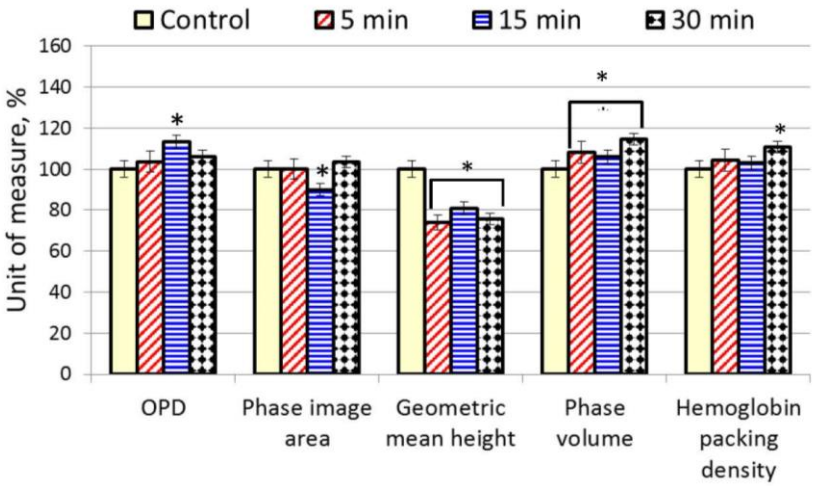

Fig. 11. RBC morphometric parameters after exposure to 1.0 mM of ATP and 100 µM of sodium nitroprusside (* – $P \leq 0.05$)

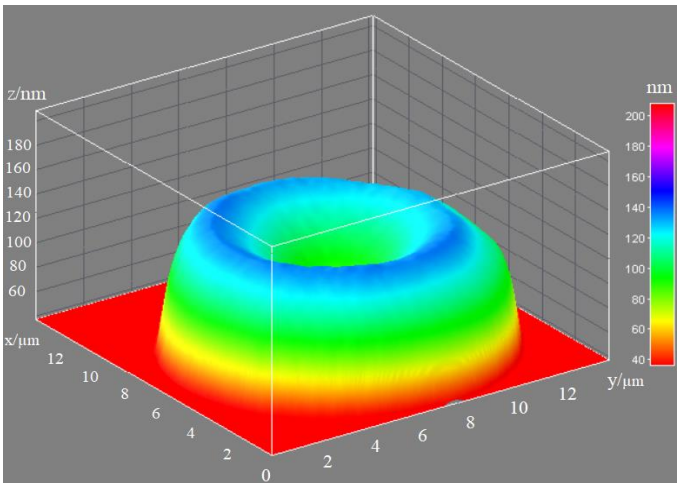

Fig. 12. Phase images of erythrocytes, taken using LIM (oX, oY: erythrocyte sizes, μm; oZ: optical path difference (OPD), nm): erythrocyte after exposure to 5.0 mM of ATP and 100 µM of sodium nitroprusside (30 minutes)

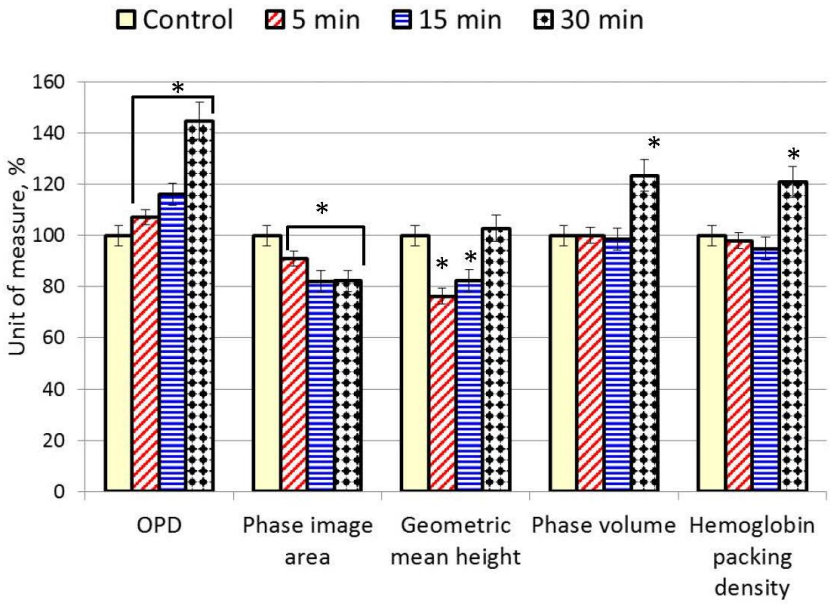

Fig. 13. RBC morphometric parameters after exposure to 5.0 mM of ATP и 100 μM of sodium nitroprusside (* – $P \leq 0.05$)

well as when exposed to 0.5 mM of ATP both in pure form and together with sodium nitroprusside (Fig. 14).

By applying electrophoresis, we isolated the following main membrane proteins of erythrocytes: spectrin, ankyrin, band 4.1 protein, band 4.2 protein, and actin (proteins that form the membrane skeleton of the erythrocyte), as well as the band 3 protein and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (proteins involved in metabolism and ion homeostasis of erythrocyte) (Giuliani *et al*., 1992) (Fig. 15).

Western blot analysis using specific polyclonal antibodies confirmed the fact that spectrin α and the anion transporter band 3 protein are indeed among the isolated proteins (Fig. 16).

After RBC proteins electrophoresis, it was found that due to exposure to different compounds, changes occur in select protein fractions of RBC membranes. Thus, in all experiments the amount of spectrin – the main protein of the cytoskeleton - has significantly reduced, especially when exposed to nitroprusside, 5.0 mM of ATP, as well as to combined action of nitroprusside and 0.5 mM of ATP.

At the same time, the effect of nitroprusside on RBCs led to a less profound decrease in other protein fractions (Fig. 17).

The exposure to 0.5 mM of ATP reduced the concentration of ankyrin to a lesser extent, and by the 30th minute of exposure to ATP, the amount of this protein did not differ from the control values. The number of select fractions was decreasing during 30 minutes of incubation: glyceraldehyde-3-phosphate dehydrogenase decreased by 76.8%, band 3 protein – by 58.3% (Fig. 18).

The exposure to 1.0 mm of ATP caused a significant decrease in all protein fractions. Thus, after the 30th minute of incubation, the amount of ankyrin decreased compared to the baseline level by 79.2%, band 3 protein – by 80.4%, and band 4.2 protein – by 78.2% (Fig. 19).

After incubation of RBCs dispensed with 5.0 mM of ATP, mostly band 3 protein and band 4.2 protein fractions changed to a larger extent. Their concentration after the 30th minute of incubation was only 22.5% and 22.9%, respectively (Fig. 20).

The exposure to ATP concentrations of 0.5 mM and 1.0 mM as well as 100 μM of sodium nitroprusside had a minor effect on quantitative changes in cytoskeleton protein fractions and RBC membrane.

When exposed to combined action of 5.0 mM of ATP and 100 μM of sodium nitroprusside for 30 minutes, the amount of band 3 protein comprised 28.1%, actin –25.4%, GAPDH – 15,2% compared to control values, respectively. Moreover, the amount of GAPDH already at the 5th minute of incubation decreased by 94% against control value (Fig. 21).

Therefore, we have shown that exposure to ATP has had profound effect on the quantitative composition of RBC membrane proteins and cytoskeleton than sodium nitroprusside or their combined action in the incubation medium.

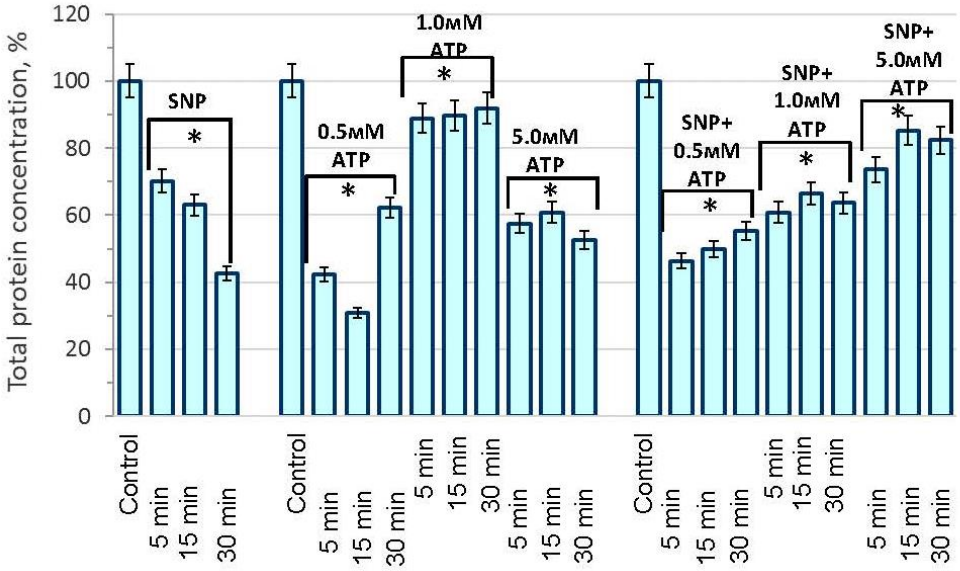

Fig. 14. Changes in the concentration of total RBC protein exposed to sodium nitroprusside and different concentrations of ATP (* – $P \leq 0.05$)

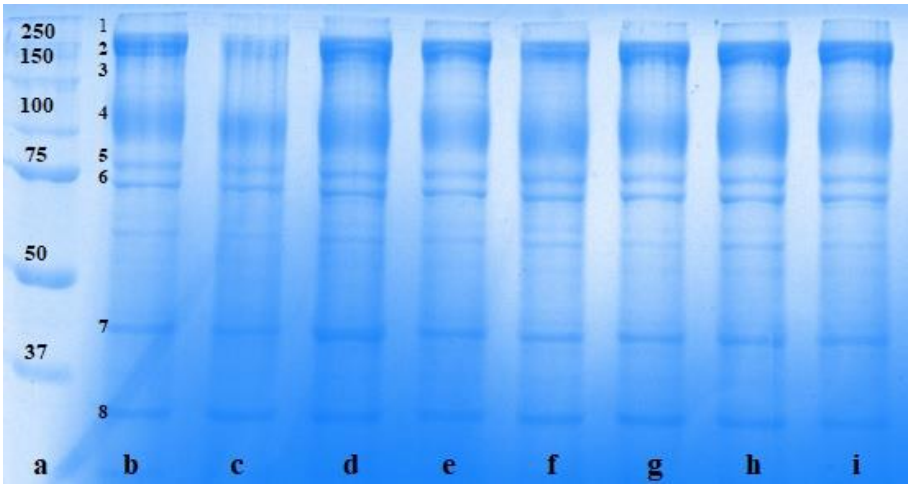

Fig. 15. Electrophoregram of cytoskeletal proteins of human erythrocytes (incubation 30 minutes): a – marker, b – control, c – SNP, d – ATP 0.5 μM; e – ATP 1.0 μM; f – ATP 5.0 μM; g – SNP+ATP 0.5 μM; h – SNP+ATP 1.0 μM; i – SNP+ATP 5.0 μM; 1 – spectrin α, 2 – spectrin β, 3 – ankyrin, 4 – band 3 protein, 5 – band 4.1 protein, 6 – band 4.2 protein, 7 – actin, 8 – GAPDH

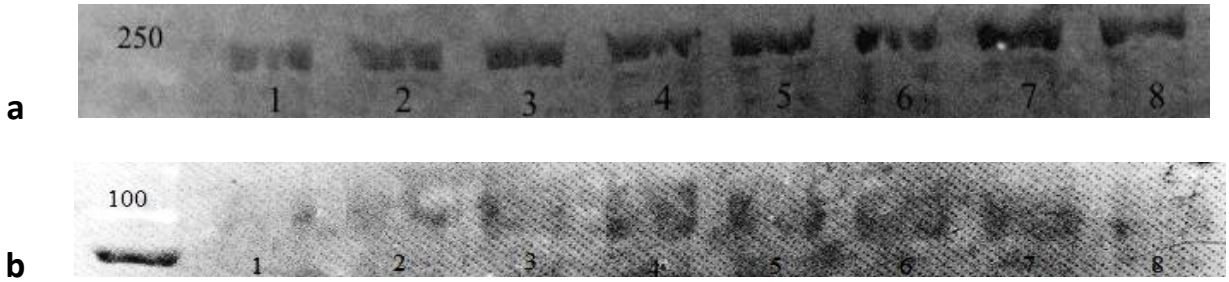

Fig. 16. Western blot analysis of spectrin α level (a) and band 3 protein (b) in erythrocyte membrane (30 minute): 1 – control; 2 – SNP; 3 – ATP 0.5 μM; 4 – ATP 1.0 μM; 5 – ATP 5.0 μM; 6 – SNP+ATP 0.5 μM; 7 – SNP+ATP 1.0 μM; 8 – SNP+ATP 5.0 μM

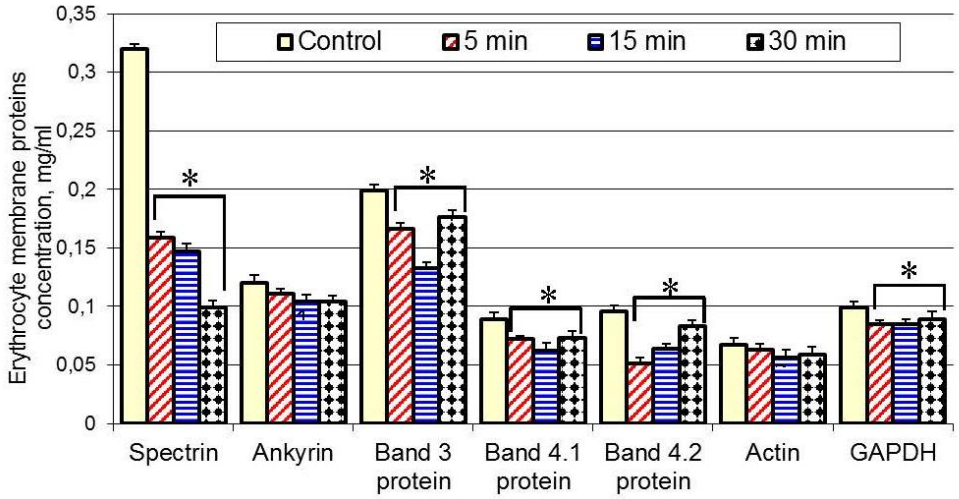

Fig. 17. Quantitative composition of RBC proteins exposed to 100 μM of sodium nitroprusside (* – $P \leq 0.05$)

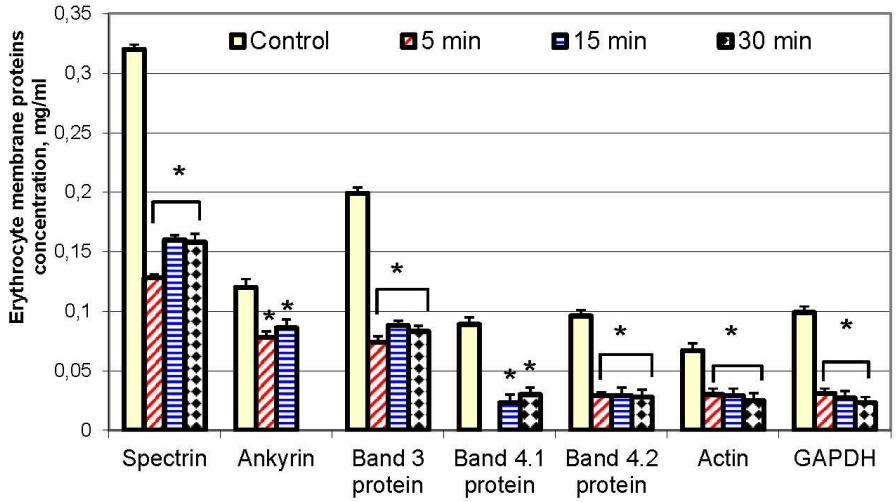

Fig. 18. Quantitative composition of RBC proteins exposed to 0.5 mM of ATP (* – $P \leq 0.05$)

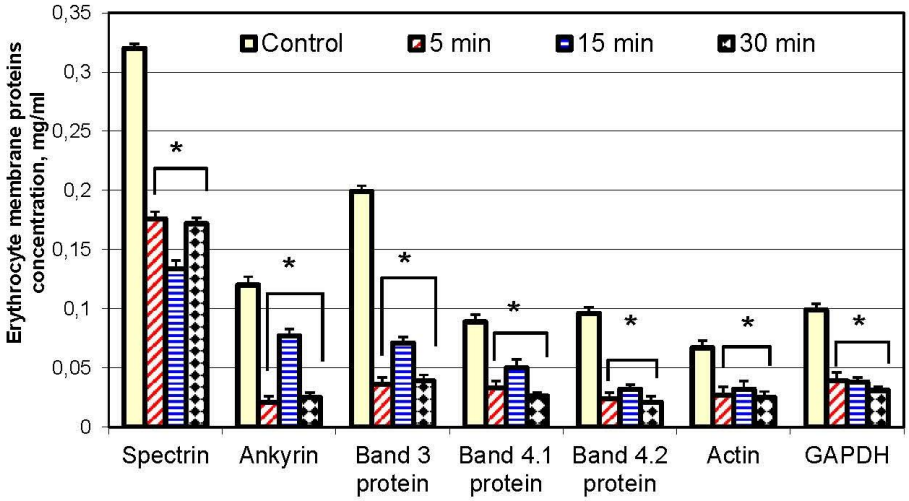

Fig. 19. Quantitative composition of RBC proteins exposed to 1.0 mM of ATP (* – $P \leq 0.05$)

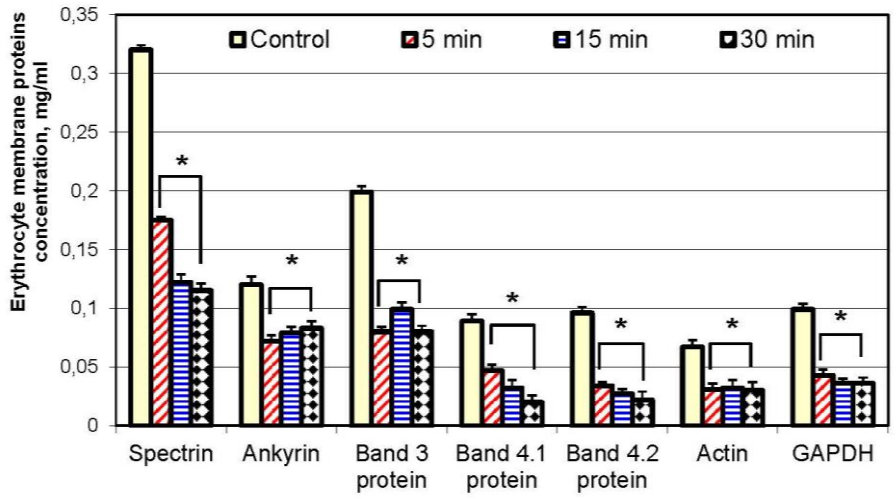

Fig. 20. Quantitative composition of RBC proteins exposed to 5,0 mM of ATP (* – $P \leq 0.05$)

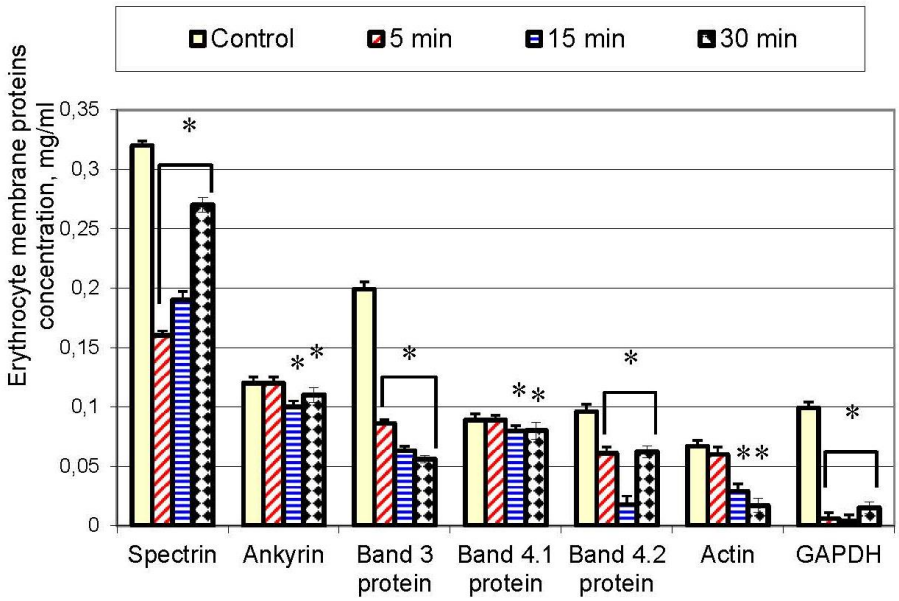

Fig. 21. Quantitative composition of RBC proteins exposed to 5,0 mM of ATP and 100 μM of sodium nitroprusside (* – $P \leq 0.05$)

Research into oxygen-binding capacity of RBC hemoglobin exposed to different ATP concentrations and sodium nitroprusside / nitric oxide donor

The study of conformation and oxygen-binding properties of hemoglobin hemoporphyrin (Hb) by means of Raman spectroscopy was carried out by looking at the changes in 1355, 1375, 1550, 1580 cm^{-1} intensity bands ratio of RBC spectra (Table 1).

RBC oxygen-binding values of healthy donors were characterized by the following parameters: the relative amount of oxyhemoglobin (oxyHb) was 0.483 ± 0.021% rel.units, Hb oxygen affinity – 1.348 ± 0.056 rel.units, the ability of Hb to bind oxygen – 1.128 ± 0.092% rel.units, the ability of Hb to discharge oxygen – 0.837 ± 0.046% rel.units.

When exposed to sodium nitroprusside, the increase in the amount of oxyHb in RBCs was observed, so at the 15th minute of incubation, this value grew by 17.2%. At the same time, the ability of hemoglobin to bind oxygen, as well as the hemoglobin oxygen affinity, decreased. The ability of hemoglobin to discharge ligands remained basically unchanged (Fig. 22).

During incubation of erythrocytes with 0.5 mM and 1.0 mM of ATP, the RBC oxygen-binding capacity changed in the same way.

When exposed to a higher concentration of ATP (5.0 mM), an increase in the relative amount of oxyHb in RBC was revealed: at the 5th minute, an increase by 29.6% was recorded, at the 15th minute - by 10.6% , at the 30th minute, the amount of oxyHb remained above the control values by 9.3%. At the same time the ability of hemoglobin to bind oxygen has significantly reduced (by 45.2%) as well as hemoglobin oxygen affinity (by 31.7%) after the 5th minute of incubation (Fig. 23).

During the next set of experiments, it was revealed that the Hb oxygen-binding capacity varies as much as possible when exposed to combined action of 100 μM of sodium nitroprusside and different ATP concentrations. Therefore, at the exposure to 0.5 mM of ATP, the relative amount of oxyHb was increasing during 30 minutes of incubation: at the 5th minute of incubation, the value rose by 47.8%, at the 15th minute – by 25.1%, at the 30th minute – by 31.9%. At the same time, the Hb ability to bind oxygen and the Hb oxygen affinity were decreasing. Similar dynamics of changes were revealed at the exposure to 1.0 and 5.0 mM of ATP together with 100 μM of sodium nitroprusside.

Therefore, we have experimentally proved the increase in the relative amount of oxyHb in RBCs at its exposure to different concentrations of ATP, sodium nitroprusside and the effect of their combined action. Moreover, the most significant increase in RBC values followed after combined action of 100 μM of sodium nitroprusside and 0.5 mM of ATP. The hemoglobin oxygen affinity was also decreasing, and the highest degree of decrease was revealed when RBCs were exposed to 0.5 mM of ATP and 100 μM of sodium nitroprusside and at the 5th minute of incubation it reached 59.2% against the control parameters. Less significant changes were observed at RBCs exposure to 0.5 mM and 1.0 mM of ATP without sodium nitroprusside.

Table 1

Raman RBC intensities ratio characteristics

Raman intensities ratio	Type of oscillations	Hb state characterisation
$I_{1375}/(I_{1355}+I_{1375})$	1355 $см^{-1}$ – pyrroles oscillations in deoxyhemoglobin molecules; 1375 $см^{-1}$ – pyrroles oscillations in oxyhemoglobin molecules; 1550 $см^{-1}$ – oscillations of methine groups between pyrroles in molecules; 1580 $см^{-1}$ – oscillations of methine groups between pyrroles in molecules	Amount of oxyhemoglobin
I_{1355}/I_{1550}		Hemoglobin's ability to bind oxygen
I_{1375}/I_{1580}		Hemoglobin's ability to discharge oxygen
$(I_{1355}/I_{1550})/(I_{1375}/I_{1580})$		Hemoglobin oxygen affinity

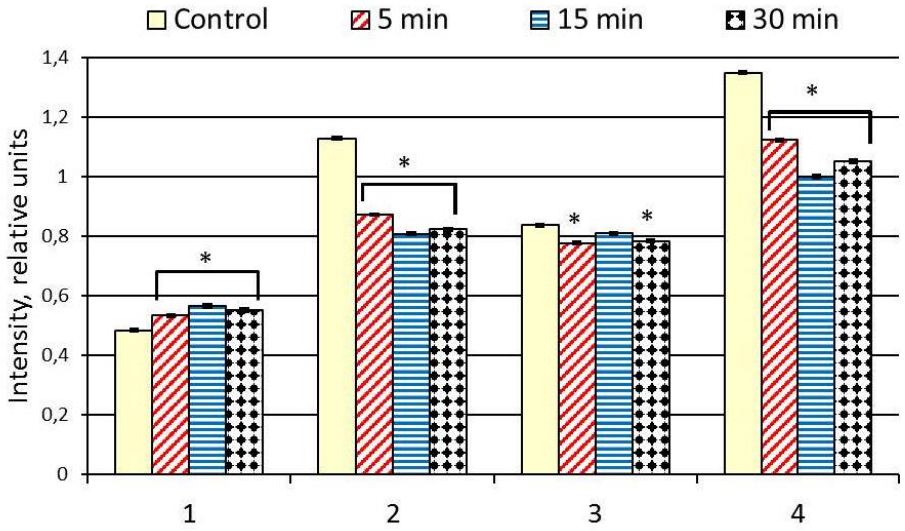

Fig. 22. Values of RBC's Hb oxygen-binding ability exposed to 100 μM of sodium nitroprusside: 1 – relative amount of oxyHb, 2 – Hb ability to bind O_2, 3 – Hb ability to discharge O_2, 4 – Hb O_2 affinity (* – $P \leq 0.05$)

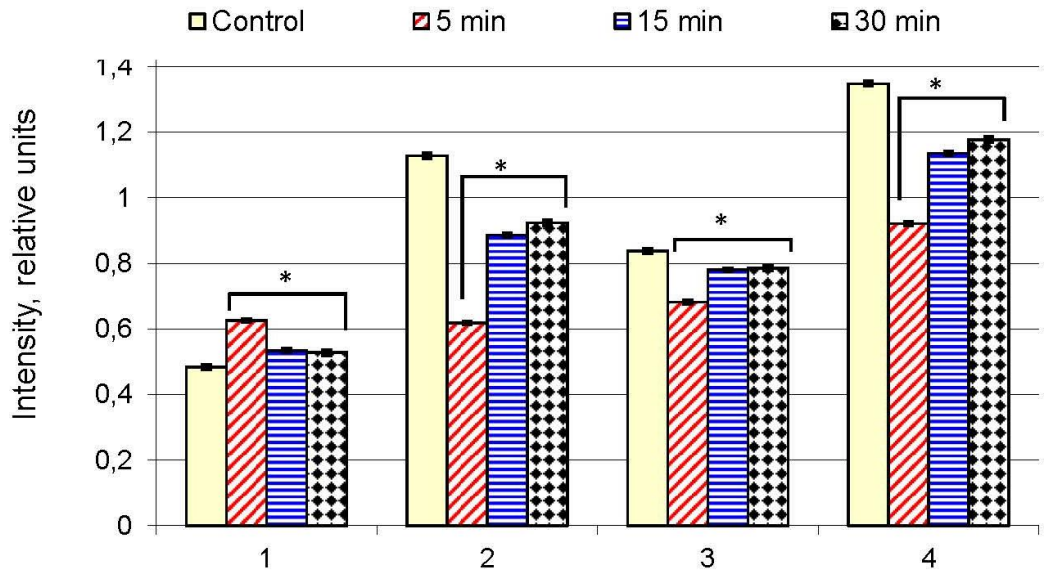

Fig. 23. Values of RBC's Hb oxygen-binding capacity when exposed to 5,0 mM of ATP: 1 – relative amount of oxyHb, 2 – Hb ability to bind O_2, 3 – Hb ability to discharge O_2, 4 – Hb O_2 affinity ($P \leq 0,05$)

Discussion

As already noted, that exogenous compounds play an important role in the regulation of hemostasis by acting both on vascular tone and on the function of blood cells themselves. It is proved, that ATP and nitric oxide NO (II) produce a relaxing effect on blood vessels, changing the contractile activity of smooth muscle cells. By acting on endothelial cells, ATP can activate purinergic receptors on their surface, which in turn enhances incoming Ca^{2+}currents, endothelial NO synthase production, and NO synthesis, which diffuses into smooth muscle cells, stimulating vasodilation and improves blood supply to tissues (Simmonds *et al*., 2014; Litvinov & Weisel, 2017). However, the effect of these compounds on the RBC properties, performing the most important oxygen transport function of cells, has been understudied. It is logical to assume that when ATP and NO (II) are in the blood channels in high (typical for pathological conditions) concentrations, they produce a significant effect on the RBC morphometric and oxygen-binding properties, in particular, through purinergic receptors (Crawford *et al*., 2004; Mozar *et al*., 2016; Knight, 2009).

Initially, we identified a number of differences in the functional response of RBCs upon their exposure to different ATP concentrations in samples. Upon exposure to small concentrations of ATP (0.5 and 1.0 mM), the RBC's physical height decreased, and the RBC phase image area increased. The RBC phase volume and the density of distribution of Hb molecules at small concentrations of ATP also decreased by the 30th minute of incubation. A high concentration of ATP (5.0 mM) resulted in an increase in the phase volume and RBC Hb packing density by the 30th minute of incubation. At the same time, the RBC phase image area remained unchanged, and the RBC geometric height was decreasing throughout the incubation time, and reached its maximum already at the 5th minute.

Therefore, small concentrations of extracellular ATP cause a change in the RBC morphology, aimed at improving the passage of cells through small vessels and capillaries (makes cells flatter), increasing the contact area of oxygen molecules with the RBC membrane and redistribution of Hb molecules within the RBC. Probably the decrease in the Hb packing density in the RBC may change Hb perimembrane and cytoplasmic fractions ratio, which plays an important role in the binding and transport of oxygen molecules through the blood channels (Steinberg *et al*., 2009; Maksimov *et al*., 2019). Higher concentrations of ATP led to a rapid decrease in RBC height and slower redistribution of Hb molecules. It can be assumed that a high concentration of ATP leads to a change in the RBC ion balance, which causes a sharp change in the RBC geometry and its phase profile. The slower process of Hb redistribution, probably associated with an increase in the concentration of Ca^{2+} in the cell and its interaction with Hb (Mohanty *et al*., 2014; Bogdanova *et al*., 2013).

The change in cell morphology is closely related to the structure of RBC membrane and cytoskeleton. Thus, in the RBC membranes after exposure to 0.5 mM of ATP at the 15th minute of incubation, the maximum decrease in the concentration of total protein was observed, by the 30th minute the values remained significantly below control level. Incubation of RBC membranes with 1.0 mM of ATP also led to a decrease in the concentration of total protein throughout the incubation time, however, the observed effect is less evident. In addition, small concentrations of ATP had the strongest effect on the amount of spectrin and actin, band 3 protein, band 4.1 protein, band 4.2 protein concentrations also decreased. Ankyrin content decreased briefly against the control values, returning to the initial value at the 30th minute. Also, by the 5th minute of incubation, the GAPDH concentration sharply decreased. Incubation of 5.0 mm of ATP with RBC membranes also led to a decrease in the concentration of total protein by almost 2 times, while a significant decrease in spectrin and actin in RBC membranes was revealed. The decrease in RBC height (5th minute) was accompanied by a maximum decrease in the amount of ankyrin, band 3 protein and actin. A prolonged decrease in the content of spectrin, GAPDH, band 4.1 protein and band 4.2 protein was observed during 30 minutes of incubation with ATP.

The obtained results confirm that changes in the RBCs geometry are accompanied by rearrangements of the RBC membrane and cytoskeleton. Moreover, the duration and intensity of the response to this effect depends on the ATP concentration impacting the RBC purinergic receptors. Thus, ATP is able to stimulate the reduction of spectrin-actin complexes that form a protein framework on the cytoplasmic surface of membranes, or transform spectrin dimers into tetramers, which also affects the ability of spectrin to form complexes with actin.

Also, anchor proteins (ankyrin, band 4.1 protein) participate in the regulation of the RBC shape, stability and deformability, which ensure the attachment of the cytoskeleton to the membrane through band 3 protein, as well as stabilize interaction of spectrin with actin when forming the main binding components of the RBC cytoskeleton. Kinase phosphorylation of band 4.1 protein and band 3 protein may relax the spectrin cytoskeleton and form a more plastic membrane structure, increasing the cell deformability as a whole. band 4.2 protein interacts with both band 3 protein and ankyrin, and a decrease in its concentration leads to the increased viscosity of the RBC membrane. GAPDH interacts specifically with band 3 protein and takes a significant part in the membrane-mediated exchange of Cl^- and HCO_3^- to form a protonated Hb form, which leads to a decreased Hb oxygen affinity (Revin *et al*., 2019; Muravyov *et al*., 2017). The decreased concentration of membrane-associated GAPDH may be attributed to phosphorylation of band 3 protein, decay of the BP3- GAPDH complex and release of GAPDH, which causes destabilization of the RBC membrane (Sirover, 2017).

Upon exposure to low concentrations of ATP, a slight increase in the Hb complexes with oxygen was recorded, while the Hb ability to bind oxygen and Hb oxygen affinity reduced significantly. This effect has been observed from the 5th minute of incubation with 0.5 mM of ATP, whereas 1.0 mM of ATP dispensed revealed a gradual change that reached its maximum by the 30th minute. Upon exposure to 5.0 mM of ATP, the maximum effect on the RBC oxygen transport properties was observed at the 5th minute of cell incubation: the relative number of oxyHb complexes increased briefly, while the Hb oxygen affinity, the Hb ability to bind and discharge oxygen decreased. By the 30th minute, the RBC oxygen transport parameters increased slightly, but remained below the control values.

It is likely that different concentrations of extracellular ATP have a mediated effect on both Hb molecule protein part and the conformation of the heme hemoporphyrin macrocycle. Firstly, the increased number of oxyHb complexes is characterized by hemoporphyrin conformational changes, in which the iron atom after oxygenation enters the porphyrin coordination cavity and is located centrosymmetrically. The structure of Hb protein is such that it shields all other molecules present in the blood from the approach to the iron atom, and timely regulates its donor-acceptor properties. Secondly, when oxygen is attached to Hb, a «heme-heme» cooperative interaction is realized, facilitating the subsequent attachment of oxygen molecules.

Thirdly, the revealed decrease in the Hb ability to bind oxygen and the decrease in the Hb oxygen affinity can probably be attributed to the predominance in hemoporphyrin molecules with stretched and deformed methine groups between pyrroles, which complicates the attachment of oxygen to the iron atom due to the spatial location of iron and additional stabilization bonds. It can be assumed that these conformational properties of Hb are linked with the formation of protonated Hb or the increase in the proportion of the perimembrane deoxygenated fraction of molecules involved in the formation and cytoskeleton maintenance stability, as well as in the mechanisms of enzymatic catalysis and intracel-

lular energy transformation (Revin *et al*., 2017; Kovalenko *et al*., 2015; Wang & Sluyter, 2013).

It is the activation of purinergic receptors on the RBC surface that can be the cause of the changes in the cell properties that we have been describing. Moreover, different concentrations of ATP have different effects on the RBC morphometric characteristics, while changes in the membrane and cytoskeleton, as well as RBC oxygen transport function have a number of similar features.

It is known that activation of RBC purinergic receptors leads to the formation of non-selective ion pores, membrane depolarization, and increases intracellular Ca^{2+}. Prolonged stimulation of receptors causes an increase in the content of negatively charged phosphatidylserine on the outer membrane surface, the formation of ROIs, apoptosis (Faulks *et al*., 2016; De Marchi *et al*., 2016).

The increase in Ca^{2+} in the RBC cytosol triggers various signaling pathways. In particular, certain enzymes are activated (protein kinases phosphatases, phospholipases), which are involved in the regulation of RBC membrane elasticity and cell deformability in general, due to phosphorylation of cytoskeletal proteins) and changes in membrane permeability. Activation of this regulatory cascade leads to dissociation of membrane cytoskeleton proteins and increased RBCs elasticity. At the core of another signaling pathway is the complex «Ca^{2+}-calmodulin», which indirectly bears on the activity of a number of channels and ATP-ases, as well as through interaction with band 4.1 protein, can play an important role in stabilizing other proteins, i.e., perform a chaperone-like function in mature RBCs. Separately, it should be noted that band 3 protein contains binding centers for both GAPDH and perimembrane Hb. And when this protein is phosphorylated, there occurs the redistribution of RBC Hb fractions, which leads to the change in the RBC oxygen transport function (Borovskaya *et al*., 2010; Rodnenkov *et al*., 2005).

It is known that NO produce a complex effect on the human circulatory system, serving, in particular, as a link in the purinergic signaling system. NO is not only synthesized in vascular cells, producing a dilated effect on their smooth muscles, but is also released into the blood channels, partially penetrating red blood cells and binding to hemoglobin (Simmonds *et al*., 2014).

To study the effect of NO on RBC morphometric, oxygen transport and membrane characteristics, 100 μM of sodium nitroprusside (SNP) was dispensed into the incubation medium. During the experiment, a change in the RBC morphometric parameters was revealed. It is important to emphasize that in the first 5 minutes of incubation of red blood cells exposed to SNP we have detected a maximum decrease in the RBC physical/geometric height. This is probably a rapid response of cells to a local increase in nitric oxide in the incubation medium. By the 30^{th} minute of incubation, we observed the increase in the phase image area of the majority of red blood cells in the samples, while the height of the cells remained below the control values, and the density of Hb distribution increased.

Therefore, when exposed to SNP, the RBC phase image area increased and their height decreased, which facilitated the passage of cells through narrow capillaries and increased the area of interaction between the membrane, oxygen and NO. At this, we observed redistribution of RBC Hb molecules, which could be accompanied by the formation of complexes with NO, as well as specific molecular associates (Shvarts *et al*., 2016; Maksimov *et al*., 2005).

It is logical to assume that RBC membrane proteins and cytoskeleton were impacted by SNP. The total amount of protein in the cell membranes after exposure to SNP was decreasing throughout the entire incubation time, in particular, the contents of spectrin, band 3 protein, ankyrin, band 4.1 protein, band 4.2 protein, and GAPDH decreased, while the actin concentration remained practically unchanged.

Most likely, SNP causes rearrangements in the main nodal complexes of the cytoskeleton. For example, nitric oxide is one of the initiators of the start of the guanylate cyclase mechanism in the cell, which activates specific erythrocyte membrane channels, cGMP-dependent protein kinase, phosphorylating proteins, cyclic nucleotides that regulate enzyme activity, and also inhibit GAPDH. In addition, by diffusing through the erythrocyte membrane, NO interacts with the near-membrane Hb in deoxy form (Dei Zotti *et al*., 2018; Cortese-Krott *et al*., 2018; Conran *et al*., 2004).

RBC oxygen transport function, characterized by conformational changes of hemoglobin hemoporphyrin macrocycle, also changes after exposure to SNP. A slight increase in the number of Hb complexes with oxygen was revealed, while the Hb ability to bind oxygen and the HB oxygen affinity significantly decreases. It is also known that NO can interact with oxyHb resulting in the formation of unstable radical and methHb, as well as with perimembrane deoxyHb, competing with oxygen for binding sites. Indirectly NO can bear on the conformation of the Hb heme pockets, which impacts the molecule

affinity for oxygen (Kovalenko *et al.*, 2015; Dei Zotti *et al*., 2018).

The results confirmed the effect of ATP and SNP on the RBC morphological and functional properties of. At this, their combined effect on cells also required a detailed study, as the development of many pathological conditions (ischemia, hypoxia) is accompanied by the destruction of red blood cells and a local increase in ATP concentration in the vessels, which produces a complex effect on the purinergic receptors of red blood cells and endothelium, simultaneously increasing the concentration of NO in the blood channels (Grygorczyk & Orlov, 2017; Sidorenko *et al*., 2018; Maksimov *et al*., 2005).

The combined action of SNP and 0.5 mM of ATP changed the RBC morphometric characteristics. Thus, already from the 5th minute of incubation, the RBC geometric height was decreasing, and the phase volume and Hb packaging density was increasing. By the 15th minute the RBC phase image area and the Hb molecules packing density decreased sharply, and at the 30th minute of incubation we have recorded the increase of RBC phase volume and decrease in height, and the phase image area and the Hb packing density did not differ from the control samples.

The concentration of total protein in RBC membranes varied significantly after exposure to 0.5 mM of ATP and SNP. The spectrin concentration decreased sharply during 15 minutes of incubation, and began to gradually increase at the 30th minute, however, without reaching the control values. At the same time, the band 3 protein concentration also fell below the control values.

The content of actin in the samples significantly decreased from the 15th minute of incubation. There was also a decrease in ankyrin, band 4.2 protein and GAPDH.

RBC oxygen transport function has reached its maximum change at the 5th minute of incubation: the relative number of oxyHb complexes increased by 1.5 times, at the same time, the Hb ability to bind oxygen and the affinity of molecules for oxygen decreased, while the Hb ability to discharge oxygen slightly increased. From the 15th minute, the changes became less obvious, and the Hb ability to discharge oxygen fell against control samples.

Therefore, the decrease in the RBC height and increase in the Hb packing density at the 5th minute of incubation with 0.5 mM of ATP and SNP was accompanied by a decrease in the concentration of spectrin, band 3 protein and ankyrin in the RBC membrane. At the same time, the number of oxyHb complexes reached the maximum increase, the Hb ability to discharge oxygen also grew, and the ability to bind oxygen fell. Probably, the increase in the concentration of ATP and NO in the incubation medium led to the activation of purinergic receptors on the surface of red blood cells and increased membrane permeability to gas molecules.

At the 15th minute, the RBC geometry undergoes a number of changes, in addition to its height, the RBC phase image area decreases, and the Hb packaging density remains below the control values. There is a significant decrease in ankyrin and GDP in the RBC membrane, the amount of actin and band 4.2 protein decreases. The conformation of hemoporphyrin undergoes changes that impair the Hb ability to discharge oxygen.

After exposure to SNP and 1.0 mM of ATP, RBC height decreased and the RBC phase volume increased from the 5th minute and throughout the incubation time. At the 15th minute, a short decrease in RBC phase image cell area occurred, by the 30th minute – the increase in the RBC Hb molecules packing density. The concentration of total protein in the RBC membranes was decreasing throughout the incubation time. At the same time, the amount of spectrin in the RBC membrane decreased throughout the incubation time by almost 2 times, the amount of actin did not change. The concentration of ankyrin, band 4.2 protein and GAPDH also decreased. The amount of band3 protein in the samples decreased briefly at the 5th minute of incubation. In addition, incubation of erythrocytes with 1.0 mM of ATP and SNP resulted in the increased oxide concentration during the 30th minute of incubation. At this, the Hb ability to bind oxygen and the Hb oxygen affinity lessened significantly.

After exposure to SNP and 5.0 mM of ATP from the 5th minute of incubation and throughout the experiment, the RBC phase image area was decreasing. At the same time, the geometric height was going within 15 minutes. By the 30th minute of incubation, the RBC phase volume and the Hb molecules packing density were significantly growing up compared to the control sample. The concentration of total protein in RBC membranes fell minimally in a set of experiments with ATP and SNP. At the 5th minute of incubation, the maximum of prolonged changes in the concentration of spectrin (decrease). At the same time, the concentration of actin was falling, starting from the 15th minute, reaching its maximum at the 30th minute. Throughout the incubation time, the amount of band 4.2 protein (maximum at the 15th minute) and GAPDH was decreasing. RBC oxygen transport function was changing

throughout the incubation time. So the concentration of RBC oxyHb complexes was growing, the Hb ability to bind oxygen and its affinity for oxygen lessened. The Hb ability to discharge oxygen was also decreasing, but more profoundly than after exposure to lower concentrations of ATP.

Conclusion

Following the objective of the study, we have revealed that activation of the RBC purinergic signaling pathway has a regulatory effect on oxygen transport and morphometric parameters of cells. Firstly, after exposure to ATP, which is the main activator of the RBC purinergic signaling pathway, we have observed a weakening effect on the Hb ability to bind oxygen, with a slight increase in the number of oxyHb complexes. Secondly, when exposed to ATP, the content of the main structural and regulatory proteins of the cytoskeleton has profoundly decreased. This process is accompanied by the change in the geometry of red blood cells, which improves the passage of cells through the capillaries, as well as increases the area of contact of oxygen with the RBC membrane. The existence of such mechanism is recounted in numerous research papers (Maksimov *et al*., 2019; Muravyov *et al*., 2017; Kovalenko *et al*., 2015; Rodnenkov *et al*., 2005). Another proof to our assumption is the results of researches by a large number of scientists, whose works show that activation of purinergic receptors and the increase in the intraerythrocytic calcium lead to changes in the ionic balance of cells, depolarization of their membranes, development of oxidative stress and activation of several protein kinases that initiates the relaxation of spectrin cytoskeleton and the formation of a more plastic membrane structure (Yawata, 2003; Bennett, 1990; Mohandas & Gallagher, 2008). Thirdly, the combined action of SNP and 0.5 mM of ATP had a profound effect on the conformation of hemoporphyrin molecules, which led to a sharp increase in the number of oxyHb complexes, while the hemoglobin oxygen affinity and the distribution density of its molecules decreased. The RBC membrane-cytoskeletal component also underwent changes: the membrane protein content decreased, and red blood cells became more compact. It should be noted that after exposure to SNP and 5.0 mM of ATP, there have been changes in the protein composition of the RBC membrane: the amount of spectrin, band 4.2 protein, actin and GAPDH decreased, while the packing density of RBC Hb molecules increased. The content of band 4.1 protein, involved in maintaining the shape of cells and carries out multiple protein interactions, decreased (Bogdanova *et al*., 2013; Wang & Sluyter, 2013). Therefore, by activating purinergic receptors, ATP produces a stronger effect on the RBC membrane-cytoskeletal component through the initiation of purinergic extracellular cascade of reactions. On the other hand the combined action of SNP and ATP has an evident effect on the RBC oxygen transport functions. Probably, upon activation of purinergic receptors, NO (II) has some protective effect, inhibiting GAPDH, which is involved in the formation of the Hb protonated form with less affinity for oxygen, as well as forming specific Hb complexes, nitrosylating proteins and activating soluble guanylate cyclase, which affects the state of the cytoskeleton (Litvinov R.I. & Weisel, 2017; Sirover, 2017; Dei Zotti *et al*., 2018; Cortese-Krott *et al*., 2018).

Data availability: the data used to support the findings of this study are included within the article and the original data used to support the findings of this study are available from the corresponding author upon request (Natalia V. Gromova, e-mail: nataly_grom@mail.ru).

Conflict of interest: the authors declare that there is no conflict of interests regarding the publication of this paper.

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References

BENNET V. (1990): Spectrin-based membrane skeleton: a multipotential adaptor between plasma membrane and cytoplasm. *Physiological Reviews* **70**, 1029–1065.

BOGDANOVA A., MAKHRO A., WANG J., LIPP P. & KAESTNER L. (2013): Calcium in red blood cells-a perilous balance. *Int J Mol Sci* **14**, 9848–987221.

BOROVSKAYA M.K., KUZNETSOVA E.E., GOROKHOVA V.G., KORIAKINA L.B., KURILSKAYA T.E. & PIVOVAROV JU.I. (2010): Structural and functional characteristics of the erythrocyte membrane and its changes in pathologies of different genesis. *Bulletin of the All-Union Scientific Center of the Siberian Branch of the Russian Academy of Medical Sciences* **3**(53), 334–354.

BRAZHE N.A., BAIZHUMANOV A.A., PARSHINA E.Y., YUSIPOVICH A.I., AKHALAYA M.YA., YARLYKOVA YU.V., LABETSKAYA O.I., IVANOVA S.M., MORUKOV B.V. & MAKSIMOV G.V. (2014):

Studies of the blood antioxidant system and oxygen-transporting properties of human erythrocytes during 105-day isolation. *Human Physiology* **40(**7), 804–809.

BRAZHE A.R., BRAZHE N.A., RODIONOVA N.N., YUSIPOVICH A.I., IGNATYEV P.S., MAKSIMOV G.V., MOSEKILDE E.& SOSNOVTSEVA O.V. (2008): Non-invasive study of nerve fibres using laser interference microscopy. *Philos Trans A Math Phys Eng Sci* **366**, 3463–3481.

BRAZHE A.R., BRAZHE N.A., SOSNOVTSEVA O.V., PAVLOV A.N., MOSEKILDE E. & MAKSIMOV G.V. (2009): Wavelet-based analysis of cell dynamics measured by interference microscopy. *Computer Research and Modeling* **1(**1), 77–83.

BRAZHE N.A., BRAZHE A.R., PAVLOV A.N., EROKHOVA L.A., YUSIPOVICH A.I., MAKSIMOV G.V., MOSEKILDE E. & SOSNOVTSEVA O.V. (2006): Unraveling cell processes: interference imaging interwoven with data analysis. *J Biol. Phys.* **32**, 191–208.

BRAZHE N.A., THOMSEN K., LØNSTRUP M., BRAZHE A.R., NIKELSHPARG E.I., MAKSIMOV G.V., LAURITZEN M. & SOSNOVTSEVA O. (2018): Monitoring of blood oxygenation in brain by resonance Raman spectroscopy. *J. Biophotonics* **11**, e201700311.

BURNSTOCK G. & KNIGHT G.E. (2004): Cellular Distribution and Functions of P2 Receptor Subtypes in Different Systems. *International Review of Cytology* **240**, 31–304.

CHAFFEY N., ALBERTS B., JOHNSON A., LEWIS J., RAFF M., ROBERTS K. & WALTER P. (2003): Molecular biology of the cell. 4th edn. *Annals of Botany* **91**, 401–401.

CONRAN N., ORESCO-SANTOS C., ACOSTA H.C., FATTORI A., SAAD S.T.O. & COSTA F.F. (2004): Increased soluble guanylate cyclase activity in the red blood cells of sickle cell patients. *Br. J. Haematol.* **124**, 547–554.

CORTESE-KROTT M.M., MERGIA E., KRAMER C.M., LÜCKSTÄDT W., YANG J., WOLFF G., PANKNIN C., BRACHT T., SITEK B., PERNOW J., STASCH J.-P., FEELISCH M., KOESLING D. & KELM M. (2018): Identification of a soluble guanylate cyclase in RBCs: preserved activity in patients with coronary artery disease. *Redox Biol* **14**, 328–337.

CRAWFORD J.H., CHACKO B.K., PRUITT H.M., PIKNOVA B., HOGG N. & PATEL R.P. (2004): Transduction of NO-bioactivity by the red blood cell in sepsis: novel mechanisms of vasodilation during acute inflammatory disease. *Blood* **104**, 1375–1382.

DE MARCHI E., ORIOLI E., DAL BEN D. & ADINOLFI E. (2016): P2X7 Receptor as a Therapeutic Target. In: *Advances in Protein Chemistry and Structural Biology.* Elsevier, 39–79.

DEI ZOTTI F., LOBYSHEVA I. I. & BALLIGAND J.-L. (2018): Nitrosyl-hemoglobin formation in rodent and human venous erythrocytes reflects NO formation from the vasculature in vivo. *PLoS ONE* **13**.

DIETRICH H.H., ELLSWORTH M.L., SPRAGUE R.S. & DACEY R.G. (2000): Red blood cell regulation of microvascular tone through adenosine triphosphate. *American Journal of Physiology-Heart and Circulatory Physiology* **278**, H1294-H1298.

DODGE J.T., MITCHELL C. & HANAHAN D.J. (1963): The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Archives of biochemistry and biophysics* **100(**1), 119–130.

ELLSWORTH M.L., ELLIS C.G., GOLDMAN D., STEPHENSON A.H., DIETRICH H.H., & SPRAGUE R.S. (2009): Erythrocytes: Oxygen Sensors and Modulators of Vascular Tone. *Physiology* **24**, 107–116.

FAIRBANKS G., STECK T.L. & WALLACH D.F.H. (1971): Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10(**13), 2606–261.

FAULKS M., KUIT T.A., SOPHOCLEOUS R.A., CURTIS B.L., CURTIS S.J., JURAK L.M. & SLUYTER R. (2016): P2X7 receptor activation causes phosphatidylserine exposure in canine erythrocytes. *WJH* **5**, 88.

GRYGORCZYK R. & ORLOV S.N. (2017): Effects of Hypoxia on Erythrocyte Membrane Properties–Implications for Intravascular Hemolysis and Purinergic Control of Blood Flow. *Front. Physiol.* **8(**1110).

GIULIANI A., MARINI S., FERRONI L., CAPRARI P., CONDÒ S.G., RAMACCI M.T. & GIARDINA B. (1992): A monoclonal antibody monitoring band 3 modifications in human red blood cells. *Molecular and cellular biochemistry* **117**(1), 43–51.

HEUKESHOVEN J. & DERNICK R. (1985): Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* **6**(3), 103–112.

JENSEN F.B. (2009): The dual roles of red blood cells in tissue oxygen delivery: oxygen carriers and regulators of local blood flow. *Journal of Experimental Biology* **212**, 3387–3393.

KNIGHT G.E. (2009): Purinergic Receptors. In: *Encyclopedia of Neuroscience*, Elseiver, 1245–1252.

KOVALENKO S.S., YUSIPOVICH A.I., PARSHINA E.YU. & MAKSIMOV G.V. (2015): Role of Purinergic Receptors of Erythrocytes in the Regulation of Conformation and Oxygen- and NO-Transporting Capacity of Hemoglobin, *Bull Exp Biol Med* **159**, 213–216.

LAEMMLI U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227(**5259), 680.

LITVINOV R.I. & WEISEL J.W. (2017): Role of red blood cells in haemostasis and thrombosis. *VOXS* **12**, 76–183.

LOWRY O.H., ROSEBROUGH N.J., FARR A.L. & RANDALL R.J. (1951): Protein measurement with the Folin phenol reagent. *Journal of biological chemistry* **193(**1), 265–275.

LUNEVA O.G., SIDORENKO S.V., MAKSIMOV G.V., GRYGORCZYK R. & ORLOV S.N. (2015): Erythrocytes as regulators of blood vessel tone. *Biochem. Moscow Suppl. Ser. A 9*, 161–171.

MAKSIMOV G.V., MAKSIMOVA N.V., CHURIN A.A., ORLOV S.N. & RUBIN A.B. (2001): Study on conformational changes in hemoglobin protoporphyrin in essential hypertension. *Biochemistry (Moscow)* **66**(3), 295–299.

MAKSIMOV G.V., RODNENKOV O.V., LUNEVA O.G., BRAZHE N.A., PARSHINA E.I., RUBIN A.B. & CHAZOV E.I. (2005): The role of plasmatic erythrocytic membrane in formation of hypoxia in patients with chronic cardiac failure. *Ter. Arkh.* **77**, pp. 70–73.

MAKSIMOV G.V., SLATINSKAYA O.V., TKHOR E.S., ANISIMOV N.A., MAMAEVA S.N. & SHUTOVA V.V. (2019): The Role of Erythrocyte Receptors in Regulation of the Conformation and Distribution of Hemoglobin. *BIOPHYSICS* **64**, 57–61.

MIKSTACKA R., RIMANDO A.M. & IGNATOWICZ E. (2010): Antioxidant Effect of trans-Resveratrol, Pterostilbene, Quercetin and Their Combinations in Human Erythrocytes In Vitro. *Plant foods for human nutrition* **65**(1), 57–63.

MINAEV V.L. & YUSIPOVICH A.I. (2012): Use of an automated interference microscope in biological research. *Measurement Techniques* **55**, 839–844.

MITYANINA V.A., PARSHINA E.Y., YUSIPOVICH A.I., MAKSIMOV G.V. & SELISCHEVA A.A. (2012): Oxygen-binding characteristics of erythrocyte in children with type I diabetes mellitus of different duration. *Bulletin of Experimental Biology and Medicine* **153**(4), 508–512.

MOHANDAS N. & EVANS E. (1994): Mechanical Properties of the Red Cell Membrane in Relation to Molecular Structure and Genetic Defects. *Annu. Rev. Biophys. Biomol. Struct*. **23**, 787–818.

MOHANDAS N. & GALLAGHER P.G. (2008): Red cell membrane: past, present, and future. *Blood* **112(**10), 3939–3948.

MOHANTY J.G., NAGABABU E. & RIFKIND J.M. (2014): Red blood cell oxidative stress impairs oxygen delivery and induces red blood cell aging. *Front Physiol* **5**.

MOZAR A., CONNES P., COLLINS B., HARDY-DESSOURCES M.-D., ROMANA M., LEMONNE N., BLOCH W. & GRAU M. (2016): Red blood cell nitric oxide synthase modulates red blood cell deformability in sickle cell anemia. *CH* **64**, 47–53.

MURAVYOV A.V., MIKHAILOV P.V. & TIKHOMIROVA I.A. (2017): Microcirculation and Hemorheology: points of interaction., *Regional blood circulation and microcirculation* **16**, 90–100.

PARK Y., BEST C.A., AUTH T., GOV N.S., SAFRAN S.A., POPESCU G., SURESH S. & FELD M.S. (2010): Metabolic remodeling of the human red blood cell membrane. *Proceedings of the National Academy of Sciences* **107**, 1289–1294.

REVIN V.V., GROMOVA N.V., REVINA E.S., MARTYNOVA M.I., SEIKINA A.I., REVINA N.V., IMAROVA O.G., SOLOMADIN I.N., TYCHKOV A.YU. & ZHELEV N. (2016): Role of Membrane Lipids in the Regulation of Erythrocytic Oxygen-Transport Function in Cardiovascular Diseases. *BioMed Research International* **2016**, 11 pp.

REVIN V.V., GROMOVA N.V., REVINA E.S., SAMONOVA A.YU., TYCHKOV A.YU., BOCHKAREVA S.S., MOSKOVKIN A.A. & KUZMENKO T.P. (2019): The Influence of Oxidative Stress and Natural Antioxidants on Morphometric Parameters of Red Blood Cells, the Hemoglobin Oxygen Binding Capacity, and the Activity of Antioxidant Enzymes. *BioMed Research International* **2019**, 12 pp.

REVIN V., GRUNYUSHKIN I., GROMOVA N., REVINA E., ABDULVWAHID A.S.A., SOLOMADIN I., TYCHKOV A. & AND KUKINA A. (2017): Effect of hypoxia on the composition and state of lipids and oxygen-transport properties of erythrocyte haemoglobin. *Biotechnology & Biotechnological Equipment* **31**, 128–137.

RODNENKOV O.V., LUNEVA O.G., ULYANOVA N.A., MAKSIMOV G.V., RUBIN A.B., ORLOV S.N. & CHAZOV E.I. (2005): Erythrocyte membrane fluidity and haemoglobin haemoporphyrin conformation: features revealed in patients with heart failure. *Pathophysiology* **11**(4), 209–213.

SCHINDELIN J., ARGANDA-CARRERAS I., FRISE E., KAYNIG V., LONGAIR M., PIETZSCH T., PREIBISCH S., RUEDEN C., SAALFELD S., SCHMID B., TINEVEZ J.-Y., WHITE D. J., HARTENSTEIN V., ELICEIRI K., TOMANCAK P. & CARDONA A. (2012): Fiji: an open-source platform for biological-image analysis. *Nature methods* **9**(7), 676–682.

SIDORENKO S.V., LUNEVA O.G., NOVOZHILOVA T.S., ALEKSEEVA N.V., RODNENKOV O.V., DEEV L.I., MAKSIMOV G.V., GRYGORCZYK R. & ORLOV S.N. (2018): Hemolysis and ATP Release from Human and Rat Erythrocytes under Conditions of Hypoxia: A Comparative Study. *Biochem. Moscow Suppl. Ser. A 12*, 114–120.

SIMMONDS M.J., DETTERICH J.A. & CONNES P. (2014): Nitric oxide, vasodilation and the red blood cell. *Biorheology* **51**, 121–134.

SIROVER M.A. (2017): Moonlighting GAPDH and the Maintenance of DNA Integrity. In: *Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH).* Elsevier, 97–111.

SHVARTS Y.S., KRUCHININA M.V., TIMOFEEVA M.M., RUDINA M.I., DOLGANOVA O.M., GROMOV A.A., BAUM V.A. & RABKO A.V. (2016): Determining the ability of erythrocyte to generate nitric oxide in patients with cardiovascular diseases. *International Journal of Applied and Basic Research* **4-2**, 386–391.

SLUYTER R. (2015): P2X and P2Y receptor signaling in red blood cells. *Front. Mol. Biosci.* **2**.

STEINBERG M.H., FORGET B.G., HIGGS D.R. & WEATHERALL D.J. (2009): Disorders of Hemoglobin: Genetics, Pathophysiology, and Clinical Management. *2nd ed. Cambridge University Press,* Cambridge, 158–184.

TUKEY J.W. (1949): Comparing individual means in the analysis of variance. *Biometrics* **5**, 99–114.

YAWATA Y. (2003): Cell Membrane: The Red Blood Cell as a Model. John Wiley & Sons, Weinheim, Germany.

YUSIPOVICH A.I., BRYZGALOVA N.YU., PARSHINA E.YU, LOMAKIN A.G., RODNENKOV O.V., LEVIN G.G., MAKSIMOV G.V. & RUBIN A.B. (2008): Evaluation of erythrocyte shape and status by laser interference microscopy. *Bulletin of Experimental Biology and Medicine* **145(**3), 382–385.

YUSIPOVICH A.I., PARSHINA E.Y., BRYSGALOVA N.YU., BRASHE A.R., BRASHE N.A., LOMAKIN A.G., LEVIN G.G. & MAKSIMOV G.V. (2009): Laser interference microscopy in erythrocyte study. *Journal of Applied Physics* **105**(10), 102037–102047.

YUSIPOVICH A.I., ZAGUBIZHENKO M.V., LEVIN G.G., PLATONOVA A., PARSHINA E.Y., GRYGORZCYK R., MAKSIMOV G.V., RUBIN A.B. & ORLOV S.N. (2011): Laser interference microscopy of amphibian erythrocytes: impact of cell volume and refractive index. *Journal of Microscopy* **244**(3), 223–229.

WANG B. & SLUYTER R. (2013): P2X7 receptor activation induces reactive oxygen species formation in erythroid cells. *Purinergic Signalling* **9**, 101–112.