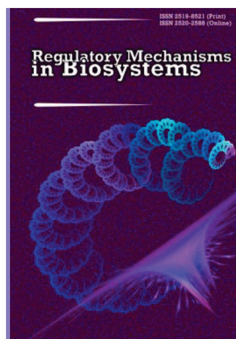

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Glycolysis process activation in preserved red blood cells by nanotechnological treatment of resuspending solutions

A. N. Belousov *, **, ***, E. I. Malygon**, ***, T. O. Kalynychenko****,
E. Y. Belousova*, **, V. V. Yavorskiy**, ***, M. Y. Anoshyna****

*Laboratory of Applied Nanotechnology of Belousov, Kharkiv, Ukraine
** Kharkiv National Medical University, Kharkiv, Ukraine
***Kharkiv Regional Center of Blood Service, Kharkiv, Ukraine
****Institute of Hematology and Transfusiology of the National Academy of Medical Sciences of Ukraine, Kyiv, Ukraine

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Laboratory of Applied Nanotechnology of Belousov, Kharkiv Medical Academy of Postgraduate Education, Nauky st., 31v, Kharkiv, 61072, Ukraine. Tel.:+38-050-915-18-89. E-mail: an.belousov2012@ukr.net

Kharkiv Regional Center of Blood Service, Klochkivska st., 366, Kharkiv, 61000, Ukraine. Tel.:+38-095-570-47-66. E-mail: e.malygon@ukr.net

Institute of Hematology and Transfusiology of the National Academy of Medical Sciences of Ukraine,Maksyma Berlynskoho st., 12, Kyiv, 04060, Ukraine. Tel.: +38-050-447-86-46. E-mail: kalynychenko_tetiana@ukr.net

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Currently, the use of nanotechnology opens up new opportunities to influence the processes of anaerobic glycolysis and the activity of hexose monophosphate reactions in preserved erythrocytes. Components containing donor red blood cells on CPDA-1 preservative were examined. Modified solutions of 0.9% NaCl and with 5% glucose were used as resuspending solutions. The solutions were treated with magnetite nanoparticles (ICNB brand) by the Belousov method. The amounts of 2,3-DPG, ATP, reduced glutathione, and glutathione peroxidase were determined by spectrophotometry. This study opens up new possibilities for increasing the shelf life and functional activity of preserved erythrocytes. The study showed a reliable increase in ATP and reduced glutathione, a decrease in 2,3-DPG and glutathione peroxidase. It was found that the activation of anaerobic glycolysis was less pronounced in tests with modified physiological saline than in tests with glucose solution. On the contrary, the pentose glucose oxidation cycle prevailed. A comprehensive analysis of the data obtained indicates the membrane-protective effect of the modified resuspending solutions. The membrane-protective effect is due to an increase in ATP and reduced glutathione, which ensures the redox potential of the cell in an equilibrium state. Magnetite nanoparticles (ICNB) change the mobility and orientation of hydrogen protons in resuspending solutions. This polarizes the aqueous sector of the erythrocyte microenvironment due to van der Waals forces, which is the main reason for activation of ATP phosphate residue hydrolysis and switching of intracellular enzymes regulating anaerobic glycolysis and pentose phosphate cycle into the active state. As a result, transmembrane metabolism and metabolism change, the energy state of erythrocytes changes, and enzymes are activated. All this has a significant impact on the energy supply of preserved red blood cells and preservation of their functional activity under storage conditions at 2 to 6 °C.

Keywords: magnetite nanoparticles; resuspending solution; preserved erythrocytes; membrane-protective effect; anaerobic glycolysis; pentose cycle.

Introduction

In the body, aerobic cells receive energy in the form of molecular oxygen (O_2). At the same time, oxygen is the source for the production of a small amount of toxic substances, the so-called reactive oxygen species (ROS) (Turrens, 2003; Hayyan et al., 2016). These compounds are strong oxidizing agents or extremely reactive free radicals that destroy cellular structures and functional molecules. Erythrocytes are particularly susceptible to damage by reactive oxygen species, which are characterized by high oxygen concentrations due to their transport function. Exposure of reactive oxygen species to unsaturated fatty acids in erythrocyte membranes leads to the formation of hydroperoxides. To protect against the formed hydroxyl and lipid radicals, erythrocytes use the antiradical system (superoxide dismutase, catalase and reduced glutathione – GSH). The antiradical system is able to inactivate ROS and eliminate the damage caused by them (Devasagayam et al., 2004; Waszczak et al., 2018).

The effective functioning of the antiradical system requires substances that ensure the maintenance of a balanced metabolism in erythrocytes. In fact, metabolism in erythrocytes is limited to anaerobic glycolysis and the hexose monophosphate pathway (HMW) (Jacobasch et al., 1982; Guitton et al., 2003). The end products of anaerobic glycolysis in the erythrocyte are adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG). ATP is the main substrate for Na^+/K^+-ATPase, which maintains the membrane potential of erythrocytes. 2,3-DPG catalyzes the dissociation reaction of oxyhemoglobin, thus providing tissues with the necessary amount of oxygen. The hexose monophosphate pathway induces the synthesis of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which in turn ensure the proper level of nicotinamide adenine dinucleotide phosphate (NADPH) synthesis in the cell. In erythrocytes the oxidative step of the hexose monophosphate pathway is the only source of NADPH. When glucose-6-phosphate dehydrogenase is insufficient, the activity of antioxidant systems weakens and hydrogen peroxide accumulates in the erythrocyte, increasing damage to its membranes and hemolysis in up to 20% of all erythrocytes (Stanton, 2012; Richardson et al., 2021).

Glutathione is one of the main intracellular low-molecular thiol-containing compounds that perform antioxidant functions, participate in maintaining the redox potential of the cell, in the detoxification system, in the synthesis of eicosanoids and in the regulation of many mechanisms of cell signaling. It neutralizes the effect of free radicals (molecules with

unpaired electrons) which actively take away the missing electron from other compounds. As a result, molecular chains are broken, the structure of substances is disturbed and reactions do not proceed (Pastore et al., 2001; Pompella et al., 2003). Those molecules that have lost an electron also become radicals. Such molecules are called oxidized, and the process of electron loss is called oxidation. A feature of glutathione, unlike other molecules in the human body, is that it quickly recovers lost electrons. By "baiting" free radicals, it gives up its electron without a fight thereby protecting cells from damage and then goes from an oxidized form (glutathione disulfide – GSSG) to a reduced form (GSH). Glutathione is mainly present in the cell in the reduced form (GSH). GSSG does not exceed 1% of its total intracellular content. Approximately 85–90% of GSH is in the cytosol. Maintaining an optimal GSH/GSSG ratio in the cell is essential for its normal functioning and survival. Therefore, it is extremely important to strictly control the system that regulates this ratio. Lack of GSH exposes the cell to the risk of oxidative damage (Novello & McLean, 1968; Lu et al., 2012).

$NADPH^+H^+$, under the action of glutathione reductase, supplies hydrogen protons for the regeneration of reduced glutathione (GSH). GSH is the most important antioxidant of erythrocytes and serves as a coenzyme in the reduction of methemoglobin to functionally active hemoglobin. Reduced glutathione is used to detoxify H_2O_2 as well as hydroperoxides, which occur during the reaction of ROS with unsaturated fatty acids of erythrocyte membranes (Halprin & Ohkawara, 1967).

In addition to glutathione reductase, selenium-containing glutathione peroxidase (GSH-Px) is an important protective enzyme (Murakami et al., 1989; Scholz et al., 1989). This enzyme catalyzes the conversion of lipid hydroperoxides to the corresponding alcohols and the reduction of hydrogen peroxide to water by reduced glutathione. The state of the glutathione system in erythrocytes significantly affects hemoglobin activity and the mechanisms of oxygen transport function regulation in blood in general (Mills et al., 1994; Richie et al., 1996; Schafer & Buettner, 2001; van Erve et al., 2013).

Currently, the use of nanotechnology opens up new opportunities to influence the processes of anaerobic glycolysis and the activity of hexose monophosphate reactions in erythrocytes. Even now with the help of nanotechnological preparations it has become possible to influence the functional activity of erythrocytes, the state of their aggregation, and to increase oxygen delivery to the tissues (Belousov, 2011). By the example of studying human erythrocytes, it was shown that magnetite nanoparticles (NPs) reliably change the polarization structure of the water sector of the cell microenvironment and, consequently, regulate the permeability of erythrocyte membranes (Belousov, 2013, 2014; Belousov et al., 2018, 2019). Resuspension of physiological saline solution treated with magnetite nanoparticles (ICNB) significantly reduces the level of primary and secondary products of lipid peroxidation and inhibits the Schiff reaction of neutral lipid peroxidation during the storage stages of preserved donor erythrocytes (Belousov et al., 2021).

Thus, the positive results of previously conducted studies were the main reason to continue studying the effect of nanotechnology methods on the functional activity of erythrocytes.

The aim of this study is to investigate the effect of nanotechnological treatment of resuspending solution on the activity of anaerobic glycolysis and hexose monophosphate reaction of preserved donor erythrocytes under storage conditions.

In order to achieve this goal, the following tasks must be solved:

– determine the amount of ATP and 2,3-DPG in erythrocytes;

– study the activity of glutathione peroxidase (GSH-Px) and changes in the content of reduced glutathione (GSH).

Materials and methods

The upgraded resuspending solutions were obtained by treating saline sodium chloride and 5% glucose solution with standardized magnetite nanoparticles (ICNB) using the Belousov method with complete removal of NPs from the solution. Magnetite NPs are synthesized by co-precipitation method. Basic physical and chemical properties of ICNB: concentration of the colloidal solution of magnetite NPs in physiological solution of NaCl is 0.0225%; theoretical osmolality of colloid solution is 500 mOsm/L; size of magnetite nanoparticles is 6–12 nm; total area of surface magnetite of nanoparticles Ss = 800–1200 m^2/g; magnetization of saturation Is = 2.15 kA/m; ζ – potential = –19 mV. Erythrocyte-containing components (ECC) obtained according to the standard method from the peripheral blood of adult healthy donors prepared on the preservative CPDA-1 were studied (n = 10, 560 studies). To study the effect of resuspending solutions on preserved erythrocytes, 0.9% NaCl solution and 5% glucose solution were used before and after treatment with magnetite nanoparticles (ICNB). The component containing red blood cells (RBCs) was divided into 4 equal aliquots. The erythrocytes from the first aliquot (SC) were resuspended in saline (control). In the second aliquot (ST), RBCs were resuspended in modified saline (test). The third aliquot (GC) of RBCs was resuspended in 5% glucose solution (control). A fourth aliquot (GT) of RBCs was resuspended in modified 5% glucose solution (test).

The 2,3-DPG content (μmol/mL) in erythrocytes was determined by the spectrophotometric method of Mranov. The 2,3-DPG content was determined by the difference in phosphorus concentration in the filtrate of erythrocyte lysate without protein and lysate adsorbed with activated carbon. First, the concentration of phosphorus was determined in ash-free erythrocyte hemolysate (total phosphorus), and then in the lysate after activated charcoal injection. Phosphorus concentration was determined using the phosphorus-molybdenum method (Mranova, 1975).

ATP content (μmol/mL) was determined spectrophotometrically in erythrocyte lysate. ATP contained in the samples phosphorylates glucose in the presence of hexokinase. The resulting glucose-6-phosphate is a substrate for the glucose-6-phosphate dehydrogenase reaction. The amount of ATP that entered the reaction was equimolar to the amount of $NADPH_2$ formed by the glucose-6-phosphate dehydrogenase reaction and was determined spectrophotometrically at 340 nm (Eschenko, 1982).

The amount of reduced glutathione (μmol/min per 1 g Hb) and glutathione peroxidase (μmol/min per 1 g Hb) was determined by spectrophotometry in a colour reaction with Ellman reagent at 412 nm (Moin, 1986).

Resuspended red blood cells were stored under hypothermia (temperature 2–6 °C) and examined for 2, 9, 16, 23, 30, 37, 44-per day from the moment of beginning of blood preservation (then – sequentially stage I, II, III, IV, V, VI, VII).

Statistical verification of the obtained data was carried out using computer programs Statistica 6.1 (StatSoft, USA). For the correct choice of parametric or nonparametric criterion in dependent or independent groups, the distribution of indicators in the groups was determined. If the Shapiro-Wilk criterion (W) $P > 0.05$, the distribution was normal. A Student's test was used to compare the SC, GC with ST and GT groups. For non-normal distribution (Shapiro-Wilk criterion $p < 0.05$), sign and Wilcoxon tests were used. If the two criteria differed on the same measure, the result was assessed using the Wilcoxon criterion. Comparison between the groups was performed separately at each stage. If one of the compared indicators had a normal distribution and the other had a non-normal distribution, the nonparametric method was used, and the result was assessed using the Wilcoxon criterion.

Results

Already at the first stage of the study, in the variants of the test where modified physiological solution was used as resuspending solution,in the preserved donor erythrocytes there were signs of activation of anaerobic glycolysis and the pentose cycle. The peculiarity of this activation was that in parallel with a significant increase in ATP there was an increase in 2,3-DPG against the background of a decrease in glutathione peroxidase (GSH-Px) (Fig. 1).

On the contrary, in the variant of tests where a modified glucose solution was used as resuspending solution, against the background of a significant increase in ATP, the increase in 2,3-DPG was insignificant. The amount of glutathione peroxidase, as in the tests with physiological solution, was significantly lower than the control (Fig. 2).

The data obtained in the first stages of the study in the variants of both tests with modified solutions indicated a significant increase in the amount of ATP in erythrocytes. The increase in the amount of ATP ensures the stability of ion transport, electrolyte balance, and the functioning of eryth-

rocyte ATPase systems. In tests where an increase in 2,3-DPG, an allosteric regulator of hemoglobin affinity for oxygen, was recorded, the dissociation curve of oxyhemoglobin shifted to the right. This potentially improves oxygen delivery by erythrocytes to tissues (Benesch & Benesch, 1967; Brewer, 1974).

On the contrary, the oxyhemoglobin dissociation curve shifted to the left in tests where there was a decrease in 2,3-DPG, which increases the oxygen-binding function of hemoglobin of preserved erythrocytes. The recorded differences in the variants of the 2,3-DPG amount test are due to the peculiarity of the primary effector effect of the polarized water spaces of the erythrocyte microenvironment on the activity of the enzymes providing allosteric control. At stage II of the study, a short-term inhibition of anaerobic glycolysis was observed in the test variant with physiological solution compared to the control (Fig. 3).

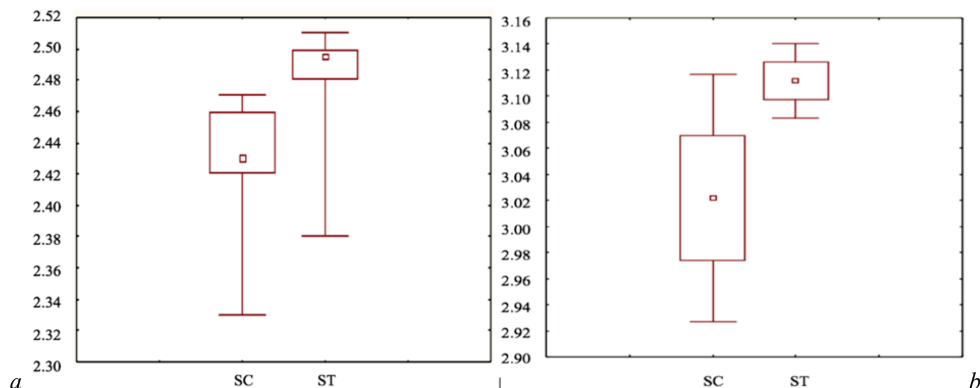

Fig. 1. Changes in intergroup difference of median ATP values (µmol/mL) and mean values of 2,3-DPG (µmol/mL) in the samples of the "Erythrocytes" component at Stage I of the study: *a* – effect of resuspending physiological saline on ATP amount (Wilcoxon test P = 0.028), small square – median, upper and lower borders of the rectangle – 25% and 75% quartiles, vertical line – minimum and maximum values, n = 10; *b* – effect of resuspending saline on the amount of 2,3-DPG (Student's t-test, P = 0. 004), small square - median, upper and lower limits of the rectangle – mean standard deviation, vertical line – mean 1.96*standard deviation, n = 10; SC – saline (control), ST – modified saline (test)

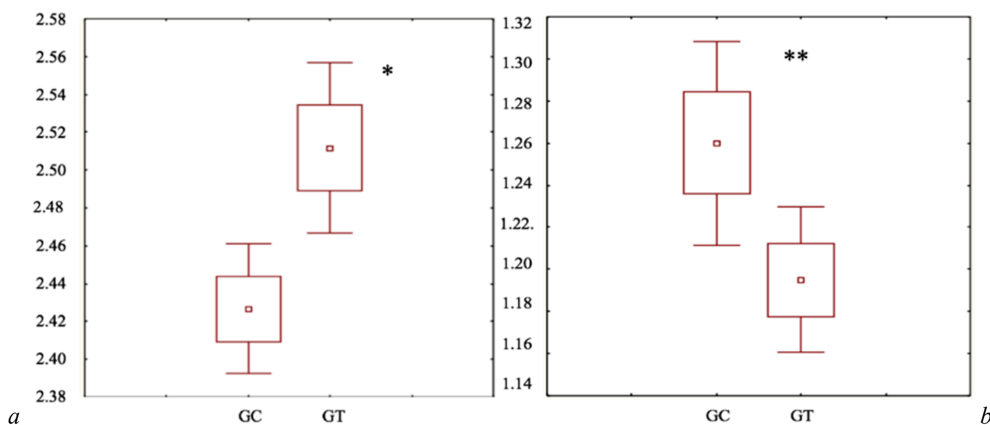

Fig. 2. Changes in intergroup difference of mean ATP values (µmol/mL) and mean GSH-Px values (µmol/min per 1 g Hb) (*b*) in the samples of the "Erythrocytes" component at Stage I of the study: *a* – effect of resuspending 5% glucose solution on ATP amount; *b* – effect of resuspending 5% glucose solution on the amount of GSH-Px; GC – 5% glucose solution (control), GT – modified 5% glucose solution (test); * – Student's t-test, P = 0.0004, ** – Student's t-test, P = 0.006; small square – median, upper and lower borders of the rectangle – mean ± standard deviation, vertical line – mean ± 1.96*standard deviation, n = 10

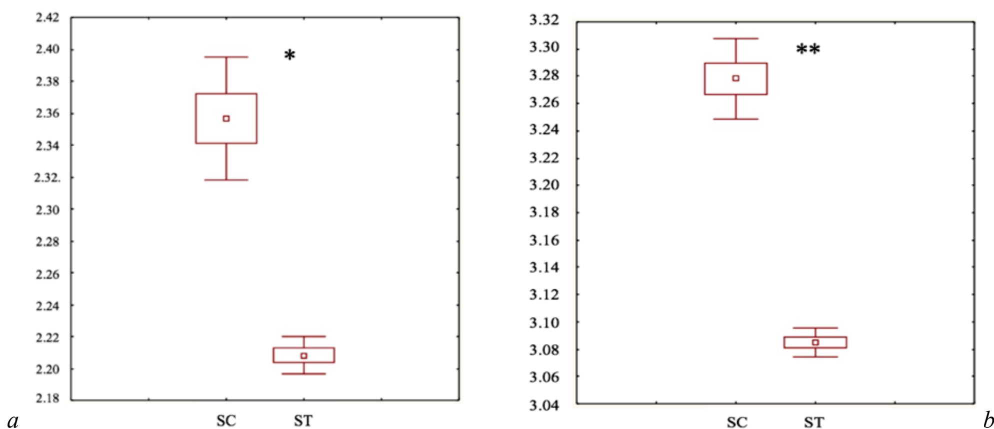

Fig. 3. Changes in intergroup difference of mean ATP values (µmol/mL) and mean 2,3-DPG (µmol/mL) in the samples of the "Erythrocytes" component at Stage II of the study: *a* – effect of resuspending physiological saline on ATP amount; *b* – effect of resuspending physiological saline on the amount of 2,3-DPG; SC – saline (control), ST – modified saline (test); * – Student's t-test, P = 0.0001, ** – Student's t-test, P = 0.000012; small square – median, upper and lower borders of the rectangle – mean ± standard deviation, vertical line – mean ± 1.96*standard deviation, n = 10

This was manifested by a decrease in the amount of ATP and 2,3-DPG against the background of the continuing activation of the pentose phosphate cycle (Fig. 4). These changes are due to the effect of selective inhibition of the Embden–Meyerhof pathway.

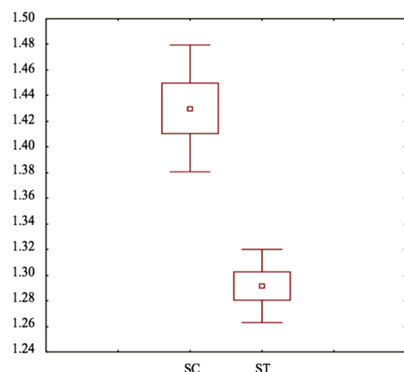

Fig. 4. Changes in intergroup difference of mean values of GSH-Px (μmol/min per 1 g Hb) in the samples of the "Erythrocytes" component at Stage II of the study; SC – saline (control), ST – modified saline (test); Student's t-test, P = 0.00051); small square – median, upper and lower borders of the rectangle – mean ± standard deviation, vertical line – mean ± 1.96*standard deviation, n = 10

In the test variant with glucose, the activation of anaerobic glycolysis, as in the first stage, was characterized by a decrease in 2,3-DPG and an increase in ATP (Fig. 5).

The amount of reduced glutathione, as compared with the control, remained significantly elevated against the background of a decrease in glutathione peroxidase (Fig. 6).

At the subsequent stages of the study, there was a significant increase in ATP and reduced glutathione, a decrease in 2,3-DPG and glutathione peroxidase in all experimental variants compared with the control (Fig.7).

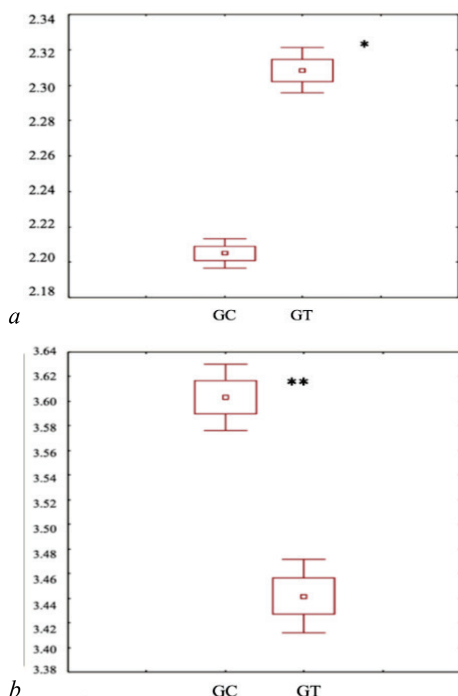

Fig. 5. Changes in intergroup difference of mean ATP values (μmol/mL) and mean 2,3-DPG (μmol/mL) in the samples of the "Erythrocytes" component at Stage II of the study: *a* – effect of resuspending 5% glucose solution on ATP amount; *b* – effect of resuspending 5% glucose solution on the amount of 2,3-DPG; GC – 5% glucose solution (control), GT – modified 5% glucose solution (test); * – Student's t-test, P = 0.00014, ** – Student's t-test, P = 0.00014; small square – median, upper and lower borders of the rectangle – mean ± standard deviation, vertical line – mean ± 1.96*standard deviation, n = 10

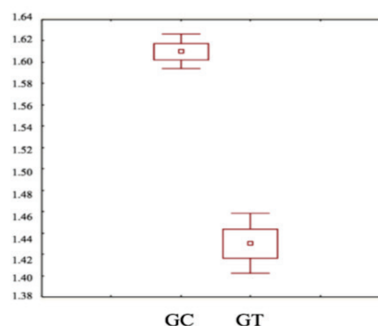

Fig. 6. Changes in intergroup difference of mean values of GSH-Px (μmol/min per 1 g Hb) in the samples of the "Erythrocytes" component at Stage II of the study; GC – 5% glucose solution (control), GT – modified 5% glucose solution (test); Student's t-test, P = 0.0002; small square – median, upper and lower borders of the rectangle – mean ± standard deviation, vertical line – mean ± 1.96*standard deviation, n = 10

Discussion

Analysis of the data obtained between the test variants showed that the severity of the changes in the studied indices differed significantly. Thus, in tests with modernized physiological saline the activation of anaerobic glycolysis was less pronounced than in tests with glucose solution. In contrast, the pentose-phosphate cycle of glucose oxidation was more pronounced. The reason for this difference is the absence of glucose in the resuspending solution as an external energy substrate for initiation in the Embden–Meyerhof pathway. In this variant, the energy deficit and metabolic disorder should have shortened the life span of the erythrocyte. However, in our case the process of glucose oxidation through activation of the pentose cycle, which clearly dominated over anaerobic glycolysis, prevailed.

The microenvironment of the cell determines the fate of pyruvate after the first ten stages of glycolysis (Melkonian & Schury, 2022). Nanotechnological treatment of resuspending solutions changes the state of the erythrocyte microenvironment, which leads to correction of metabolic processes. The possibility of differential effects on metabolic processes opens up new ways of increasing the adaptation of erythrocytes to changes in the external environment. The emergence of new methods capable of increasing the level of ATP in erythrocytes, including the use of allosteric effectors and additive solutions specific for erythrocyte and transfusate metabolites, is the main direction of optimization and correction of erythrocyte function (McMahon et al., 2021). In our case, the change in the polarization structure of water activates the cascade of enzyme systems that provide the process of G-6PDH dehydrogenation. This determines the adaptive restructuring of metabolic processes, which are primarily aimed at maintaining the structural integrity of erythrocyte membranes. This concept adds to the knowledge in the development of new methods for regulating metabolic processes in erythrocytes.

Glucose-6-phosphate dehydrogenase deficiency is known to reduce the ability of erythrocytes to resist oxidative stress (Francis et al., 2020), and ion homeostasis is controlled by energy-dependent mechanisms, which, in turn, depend on redox and energy metabolism. To mitigate oxidative stress and oxygen consumption, mature RBCs lose mitochondria and strengthen their antioxidant systems to specifically maintain haemoglobin iron in a reduced state, even in the presence of high oxygen concentrations (Yoshida et al., 2019). In addition, storage of erythrocytes in hypothermic conditions negatively affects proton pumps, disrupts the regulation of ion homeostasis.

The data obtained extend the understanding of the mechanisms of membrane-protective action of nanotechnologically modified resuspending solutions. First of all, it is associated with an increase in the amount of macroergic compound (ATP) and activation of pentose-phosphate pathway of glucose oxidation with formation of reduced form of coenzyme nicotinamide adenine dinucleotide phosphate ($NADPH_2$) with transition of oxidized form of glutathione to reduced form. The hexose monophosphate pathway is the sole route for recycling nicotinamide adenine dinucleotide phosphate (NADPH), which powers the thiol-based antioxidant system critical for homeostasis. In oxygenated erythrocytes, the activity of

the hexose monophosphate pathway is preferable because the interaction of enzymes in the Embden–Meyerhof pathway produces NADPH, which provides the reducing equivalents necessary for antioxidant systems (Timothy et al., 2021). In our case, a significant decrease in the amount of glutathione peroxidase and an increase in reduced glutathione in the erythrocytes studied indicated the transition of the antioxidant system to an active state of protection of erythrocyte membrane lipids from free-radical oxidation (Belousov et al., 2019).

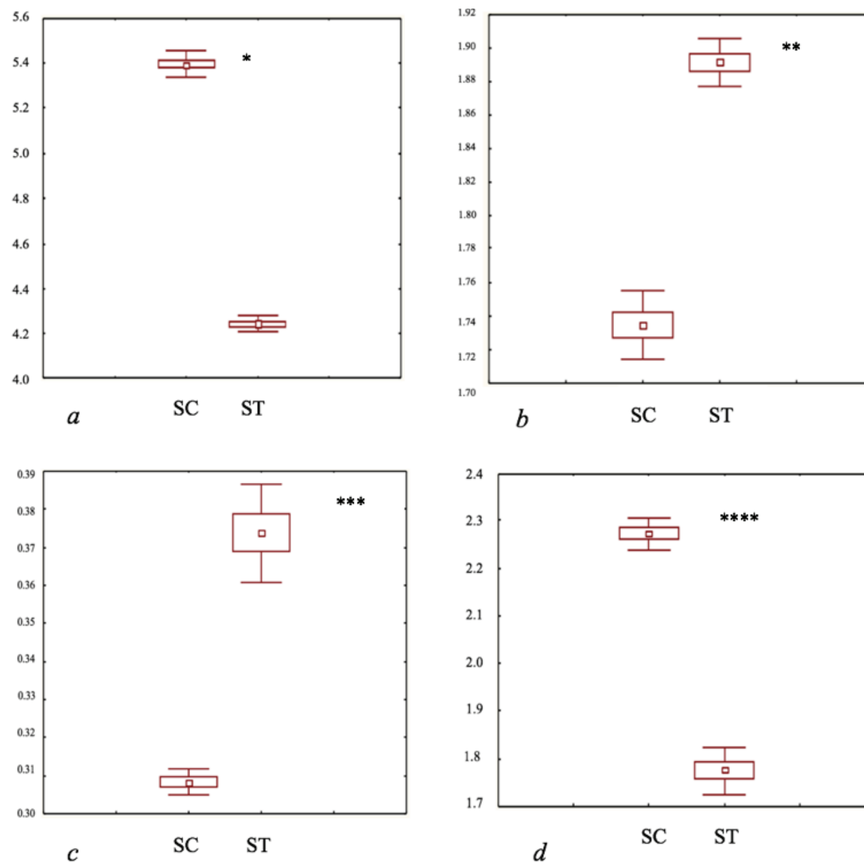

Fig. 7. Changes in intergroup difference of mean 2,3-DPG (µmol/mL), ATP values (µmol/mL), range of mean GSH values (µmol/min per 1 g Hb) and mean GSH-Px values (µmol/min per 1 g Hb) in the samples of the "Erythrocytes" component at Stage VII of the study: *a* – effect of resuspending physiological saline on 2,3-DPG amount; *b* – effect of resuspending physiological saline on the amount of ATP; (c) - effect of resuspending physiological saline on the amount of GSH; *d* – effect of resuspending physiological saline on the amount of GSH-Px values; SC – saline (control), ST – modified saline (test); * – Student's t-test, P = 1.0 x 10^{-6}, ** – Student's t-test, P = 7.0 x 10^{-5}, *** – Student's t-test, P = 1.0 x 10^{-4}, **** – Student's t-test, P = 1.0 x 10^{-5}; small square – median, upper and lower borders of the rectangle – mean ± standard deviation, vertical line – mean ± 1.96*standard deviation, n = 10

Water is a polar dielectric and can undergo the phenomenon of electric polarization, reducing its configurational and vibrational entropy. Experiments with the transfer of quantum states showed that when the electric polarization of water changes, the catalytic activity of an intracellular enzyme, enolase, involved in the regulation of the glycolysis pathway, increases (Pietkiewicz et al., 2021). The permanent magnetic field induced by magnetite nanoparticles changes the mobility and orientation of hydrogen protons in resuspending solutions. Magnetic nanoparticles have good biocompatibility with cell membranes (Agarwal et al., 2022). The introduction of nanotechnologically treated solutions into preserved donor erythrocytes polarizes the aqueous sector of the microenvironment due to van der Waals forces. This results in activation of hydrolysis of phosphate ATP residues with improvement of energy supply of erythrocytes, as well as transition to an active state of anaerobic glycolysis and pentose phosphate cycle regulating intracellular enzymes with changes in transmembrane exchange and metabolism. These changes result in increased preservation and functional activity of erythrocytes stored at 2 to 6 °C.

Conclusion

The results obtained made it possible to expand the biological application of magnetic nanoparticles on biointerfaces and in blood suspensions. Nanotechnological treatment of the resuspending solution was used to influence anaerobic glycolysis and hexose monophosphate reaction activity of preserved donor erythrocytes. Reliable increase in ATP and reduced glutathione, decreases in 2,3-DPG and glutathione peroxidase are the cumulative effect of modified resuspending solutions on preserved erythrocytes under storage conditions at 2 to 6 °C. It was found that activation of anaerobic glycolysis was less pronounced in tests with modified physiological saline than in tests with glucose solution. On the contrary, the pentose glucose oxidation cycle was predominant.

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