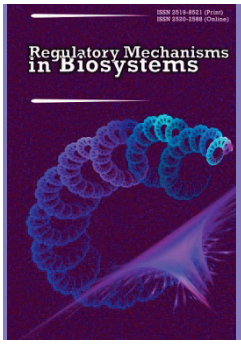

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Mechanotransduction signaling pathways of erythrocytes associated with restructuring of cell metabolism

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Erythrocytes exhibit the properties of "sensor" of mechanical tension, hypoxia and "regulator" of vascular tone. In the in vivo bloodstream, these cells are constantly exposed to flow during which they experience varying levels of shear stress and strain. In this regard, these cells have well-established signaling mechanisms, with the participation of which a chemical response to a stress factor is formed. Vibration is a factor that, depending on its own physical characteristics, combines mechanical influence with an oxidative state or hypoxia. Thus, it was of interest to investigate how erythrocytes use certain signaling pathways to maintain metabolic homeostasis under the influence of low-frequency vibration. The paper examines the effect of vibration (frequency range 8–32 Hz, amplitudes 0.50 ± 0.04 and 0.90 ± 0.08 mm) on the energy state of human erythrocytes in the absence of glucose. In this connection, the changes of intracellular ATP, 2,3-BPG and inorganic phosphate (Pi) in human erythrocytes during 3-hour vibration exposure were investigated. The activity of Na^+,K^+-ATPase was investigated as an indicator reflecting cellular needs for ATP. Cytosolic 5'-nucleotidase (cN-1A) and AMP-deaminase (AMPDA) activities were investigated as indicators of the level of catabolism of purine nucleotides. To assess the involvement of adenosine in the processes of reverse signaling through the ADORA2B – AMPK BPGM axis, the activity of ectonucleotidase (eN) was investigated. Based on the obtained experimental data, an analysis of the signal mechanisms involved in the mechanotransduction of the vibration effect was carried out. It is shown that under certain conditions of vibration exposure (frequency interval 20–32 Hz, A = 0.50 ± 0.04 mm and 12–32 Hz, A = 0.90 ± 0.08 mm) erythrocytes use signaling and metabolic pathways aimed at increasing the content of ATP, 2,3-BPG and restoration of the energy charge of cells. One of these pathways is controlled by AMP-kinase (AMPK), which in turn is a participant in the signaling cascade that begins with adenosine receptors ADORA2B. AMPK turns off consumption pathways and turns on alternative pathways for ATP regeneration and activation of 2,3-BPG formation mechanisms. These ways are aimed at overcoming the state of hypoxia. Experimental data on the participation of AMP catabolism enzymes in ATP recovery processes were analyzed.

Keywords: ATP, 2,3-BPG; Na^+,K^+-ATPase; 5'-nucleotidase; AMP-deaminase; hypoxia; adenylate metabolism; signaling mechanisms.

Introduction

Erythrocytes exhibit the properties of a "sensor" of mechanical tension, hypoxia, and a "regulator" of vascular tone (Zhang et al., 2018; Chen et al., 2023; Cilek et al., 2023). In the *in vivo* bloodstream, these cells are constantly exposed to flow during which they experience varying levels of shear stress and deformation (Zhang et al., 2018). Recently, mechanosensitive channels have been identified, such as Piezo1 (Vaisey et al., 2022; Lew, 2023), Pannexin 1 (Locovei et al., 2006; Kirby et al., 2021), receptors (GPCRs) associated with G proteins (Sridharan et al., 2012; Ellsworth et al., 2016; Ugurel et al., 2022), which can sense shear stress and affect the state of the cytoskeleton of erythrocytes. It has been shown (Ugurel et al., 2022) that the effect of mechanical stress is an increase in the level of phosphorylation of both membrane and cytoplasmic cells' proteins. In response to a hypoxic stimulus or an increase in shear stress, erythrocytes are able to export more ATP to the extracellular environment, which is involved in the regulation of vascular tone (Racine & Dinenno, 2019; Ferguson et al., 2021; Gou et al., 2021; Kirby et al., 2021; McMahon et al., 2021).

Adenosine triphosphate (ATP), which is hydrolyzed to ADP in the extracellular environment (Locovei et al., 2006; Marginedas-Freixa, 2018; Yeung et al., 2018), is a ligand for purinergic receptors of endothelial cells (Kirby et al., 2021; D'Alessandro et al., 2023). When these receptors are activated, endothelial cells produce nitric oxide, a powerful vasodilator. In addition, P2Y13 receptors that bind ADP are also exposed on the erythrocyte membrane (Montalbetti et al., 2011).

The erythrocyte membrane has the enzyme ectonucleotidase (eN, CD73), which converts AMP to adenosine and can change to a soluble state of sCD73 (Schneider et al., 2019). An increase in the amount of adenosine activates the A2B adenosine receptor (ADORA2B) and through it the ADORA2B – AMP-activated protein kinase (AMPK) – bisphosphoglycerate mutase (BPGM) signaling axis (Peng et al., 2019; Qiang et al., 2021; Misiti et al., 2022; Chen et al., 2023).

Ultimately, these mechanisms contribute to the formation of 2,3-bisphosphoglycerate (2,3-BPG), a specific erythrocyte allosteric modulator that reduces the binding affinity of hemoglobin for O_2 to counteract hypoxia (Liu et al., 2016; Peng et al., 2019; D'Alessandro & Xia, 2020; Nemkov et al., 2021). The release of ATP by erythrocytes has a regulatory effect on the erythrocytes themselves, as a type of autocrine signaling (ATP released from erythrocytes can affect the microrheology of the cells themselves). It is understood that all these processes must be regulated by well-established signaling mechanisms, in the understanding of which many gaps remain.

Vibration can be perceived directly by membrane mechanoreceptors, as shaking creates fluid flows around the cell surface. Also, the target of vibration is nanobubbles of dissolved air, which are retained on the surface of erythrocytes due to Coulomb interactions and are able to coagulate and increase in size under the action of vibration (Bunkin et al., 2011; Dotsenko et al., 2021; Gudkov et al., 2021; Takahashi et al., 2021). It is assumed that the oxygen of the bubbles is involved in the processes of oxygenation – deoxygenation with the participation of hemoglobin (Bunkin et al., 2011). Shaking the solutions causes the bubbles to collapse and release energy.

As we have shown earlier, vibration at a certain frequency, amplitude and duration leads to a decrease in the content of dissolved O_2 and CO_2 through degassing, creating a state of hypoxia (Dotsenko et al., 2021) and changing the intracellular pH. In this regard, all the signaling processes described above can be observed in cells under vibration conditions.

The paper examines the effect of low-frequency vibration (frequency range 8–32 Hz, amplitudes 0.50 ± 0.04 and 0.90 ± 0.08 mm) on the energy state of human erythrocytes. Changes in intracellular ATP, 2,3-BPG and inorganic phosphate (Pi) during 3-hour exposure to vibration were investigated as indicators of this condition. The activity of Na^+,K^+-ATPase was investigated as an indicator reflecting cellular needs for ATP. Cytoplasmic 5'-nucleotidase (cN-IA) and AMP-deaminase (AMPDA) activities were investigated as indicators of the level of catabolism of purine nucleotides. To assess the involvement of adenosine in reverse signaling processes through the ADORA2B – AMPK BPGM axis, the activity of ectonucleotidase (eN) was investigated. Based on these indicators, the signaling mechanisms of mechanotransduction of the vibration effect in human erythrocytes are analyzed.

Material and methods

The protocol of the experimental part of the study complies with the principles of biological ethics and was approved by the Local Ethics Committee of the Vasyl Stus Donetsk National University, Faculty of Chemistry, Biology and Biotechnology (Vinnytsia, Ukraine).

Fresh blood from donors of approximately the same age group and gender was used in the experiments. Erythrocytes were separated from plasma by centrifugation and washed three times with Na-phosphate buffer (0.015 M, pH 7.4) (buffer solution 1) containing 0.15 M NaCl. The total hemoglobin content in the obtained erythrocyte paste was determined by the hemoglobincyanide method.

The resulting paste of erythrocytes was introduced into the environment of the same buffer solution without glucose. The hemoglobin content in the studied suspensions was at the level of 3.02 ± 0.18 mg/mL. Suspensions were exposed to low-frequency vibration for 3 hours in the frequency range from 8 to 32 Hz (Dotsenko & Troshchynskaya, 2014, 2021; Dotsenko et al., 2021). The vibration frequency was changed in steps of 4 Hz. The amplitude of vibration (A) was maintained at the level of 0.50 ± 0.04 and 0.90 ± 0.08 mm. Vibration was carried out with the help of a vibrostand, which consisted of a generator of low-frequency sinusoidal signals, an amplifier and a vibrator that oscillates in a vertical plane with a given frequency and amplitude (Dotsenko et al., 2021). The experimental cuvette, filled with a suspension of erythrocytes, was tightly fixed vertically on the moving part of the vibrator (in this case, mechanical vibrations are transmitted to the experimental cuvette with minor power losses).

The content of the main metabolites (ATP, 2,3-BPG, inorganic phosphate (Pi)), enzyme activity (Na^+,K^+-ATPase, cytoplasmic 5'-nucleotidase (cN-1A), AMP-deaminase (AMPDA) was studied in hemolysates of erythrocytes to at the beginning of the experiment, and then every 20 minutes during the vibration exposure. The activity of the membrane-associated 5'-nucleotidase (eN) was determined in the shadows of erythrocytes. The influence of the incubation medium on all the investigated parameters was studied separately.

The activities of Na^+,K^+-ATPase, eN and cN-IA were studied using the ATP hydrolysis reaction (AMP) with subsequent determination of inorganic phosphate (Pi) (Rozhkovskij & Kresjun, 1991; Dotsenko et al., 2013; Dotsenko & Troshchynskaya, 2014). The hemolysate (suspension of ghosts) was introduced into the medium containing ATP (AMP). The composition of the medium for determining 5'-nucleotidase: 1.0 mM $MgCl_2$, 4 mM AMP, 50 mM Tris-HCl, pH 7.0. The composition of the medium for determining Na^+,K^+-ATPase: 5 mM $MgCl_2$, 120 mM NaCl, 20 mM KCl, 5 mM ATP, 50 mM Tris-HCl, pH 7.0. Enzyme activities were expressed in μM of Pi formed during 1 min, related to the amount of protein in the sample (μM/min•g of protein).

The activity of AMPDA was determined by the kinetic spectrophotometric method based on the registration of the accumulation of IMP in the deamination reaction. The composition of the medium for determining AMPDA: 0.05 M Tris HCl, pH 7.0, 150 mM KCl, 30 mM AMP. The reaction was started by adding cell hemolysate. The optical density was recorded at 285 nm for 10 min, the recording step was 1 s. AMPDA activity was expressed as μM/min•g protein using the difference in extinction for AMP and IMP equal to 0.3 (Lushchak et al., 2008).

The content of ATP, 2,3-BPG and Pi in erythrocytes was studied by the non-enzymatic method of simultaneous determination of the content of inorganic phosphate (Deryugina et al., 2018). The content of ATP, 2,3-BPG and Pi was expressed in μmol/g Hb in the sample.

A color reaction with ammonium molybdenum slime was used to determine the content of inorganic phosphate (Pn). The optical density of the solutions was recorded spectrophotometrically at a wavelength of 590 nm in cuvettes 1 cm thick. The pH content was determined using a calibration graph constructed for a standard solution of KH_2PO_4 of exact concentration (Dotsenko & Troshchynskaya, 2014).

The experiments were carried out at a temperature of 25 °C in three repetitions (n). The results were analyzed in the Statistica 8.0 program (StatSoft Inc., USA). Experimental data are presented as x ± SE (x is mean, SE is standard error). In the figures, the experimental points are connected by the regression dependence obtained using the Distance Weighted Least Squares function, P = 0.25. Three-dimensional scatter plots were used to present the obtained experimental data and identify relationships between the studied parameters. Based on the network of starting points, a surface was constructed using the method of inversely weighted distances.

Results

In the absence of glucose in the incubation environment, erythrocytes tended to experience a depletion of ATP. At the beginning of incubation, an increase in the ATP content could be registered. The presence of the ATP resynthesis phase strongly depended on the ATP content in the cell. The decrease in ATP concentration in the cell after 3 hours of incubation was usually 28–30% of the initial level. During 2 hours of cell incubation, the content of 2,3-BPG decreased by more than 30.0 ± 13.8%. Further incubation led to an increase in the content of 2,3-BPG presumably due to additional consumption of ATP. The content of Ri increased throughout the incubation period and in three hours increased by 3.0–3.2 times relative to the initial level.

The activity of Na^+,K^+-ATPase of erythrocytes under these conditions of incubation correlates with the content of intra-cellular ATP. Increasing the incubation time led to a decrease in enzyme activity by 35–40% relative to the initial level. cN activity decreased with incubation time. After 100 minutes of the experiment, cN activity decreased by 51.8 ± 10.5%. Against the background of a decrease in cN activity, AMPDA activity increased. The results of the control experiment, in which the erythrocytes were not exposed to vibration, coincide with the data obtained earlier, therefore graphical dependencies are not given.

The changes of ATP and 2,3-BPG content in cells exposed to vibration in the frequency range of 8–32 Hz, with amplitudes of 0.50 ± 0.04 and 0.90 ± 0.08 mm are shown in Figures 1, 2. It can be seen that the change in ATP concentration significantly depended on the frequency and amplitude of vibration. Thus, vibration with an amplitude of 0.50 ± 0.04 mm at a frequency of 8–16 Hz led to a gradual depletion of ATP in the cell (Fig. 1a): the decrease in the concentration of ATP in erythrocytes after 3 hours of vibration with a frequency of 8 Hz was 65.0 ± 15.8%, 12 Hz – 20.0 ± 8.9%, and 16 Hz – 12.0 ± 3.7% relative to the initial level.

Under the influence of vibration in the frequency range of 20–28 Hz (Fig. 1b), a two-phase nature of the change in ATP content was observed. Thus, vibration with a frequency of 20 Hz led to a decrease in ATP content by 75.0 ± 19.8% after 90 minutes of the experiment, after which the ATP concentration increased and reached a level 13.1 ± 3.2% lower than the initial one. Under vibration at a frequency of 24 Hz, the reduction in ATP content after 90 minutes amounted to 10.0 ± 2.4% of the initial level. At the end of the experiment, the ATP content increased and reached the control level (Fig. 1b). Exposure to vibration with a frequency of 32 Hz led to an increase in ATP concentration by 2.0–2.5 times relative to the initial level (Fig. 1c). In all cases of vibration exposure with an amplitude of 0.50 ± 0.04 mm, a direct correlation of the 2,3-BPG content with the ATP concentration in the cell was observed (Fig. 1d, 1f).

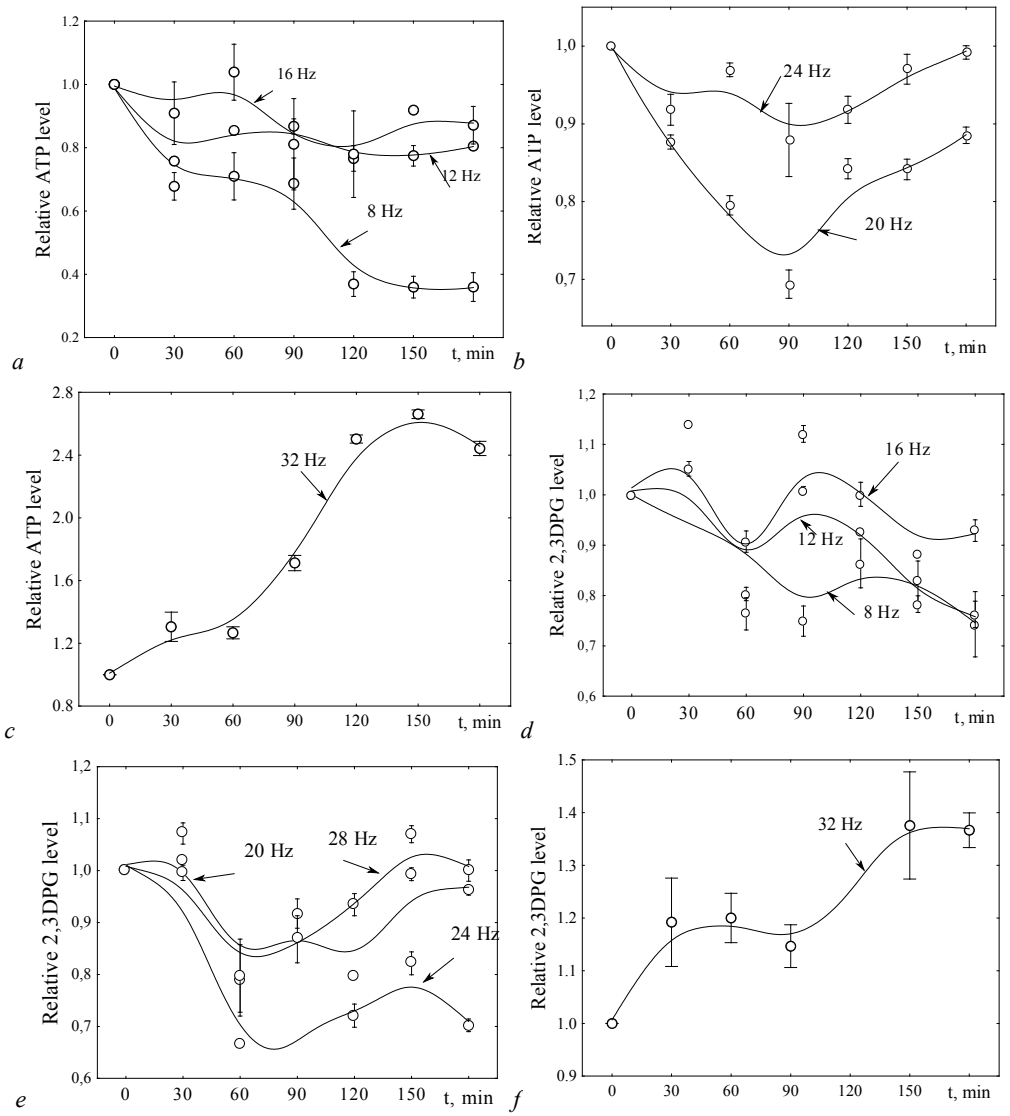

Fig. 1. ATP (*a–c*) and 2,3-BPG (*d–f*) content in erythrocytes relative to the initial level:
vibration impact parameters: frequency 8–32 Hz, A = 0.50 ± 0.04 mm; each point is represented as x ± SE, n = 3

Vibration with an amplitude of 0.90 ± 0.08 mm in the same frequency interval led to an increase in the ATP content already after 30 min of exposure (Fig. 2a, 2b). In the case of vibration with a frequency of 8 Hz, a decrease in ATP content was observed at first, and after 120 min of exposure – an increase. In the frequency range of 12–32 Hz, the ATP content after 30 min of influencing exceeded the initial level by 2.83 ± 0.25 (12 Hz), 1.60 ± 0.25 (16 Hz), 1.23 ± 0.15 (20 Hz), 1.45 ± 0.17 (28 Hz), 1.17 ± 0.10 (32 Hz) times. In the case of vibration in the frequency range of 24–32 Hz, the maximum ATP content was recorded later in time (Fig. 2b). In all cases, the content of 2,3-BPG was also directly correlated with the content of ATP (Fig. 2c, 2d).

The change in Na^+,K^+-ATPase activity in vibration experiments is shown in Figure 3a,b. Under the influence of vibration with frequencies of 8–16 Hz, A = 0.50 ± 0.04 mm, as in the control series of experiments, the change in ATPase activity was consistent with changes in the ATP content in the cell. At the beginning of the experiment, the activity of the enzyme increased by 1.80–2.31 times, but further vibration led to a gradual decrease in the activity of Na^+,K^+-ATPase (the decrease in activity was 49.9 ± 15.1% (8 Hz), 20.0 ± 12.7% (16 Hz).

During vibration with frequencies of 20–28 Hz, A = 0.50 ± 0.04 mm, the decrease in enzyme activity (Fig. 3a) occurred against the background of an increase in intracellular ATP content (Fig. 1a, 1b). Thus, under the action of vibration with a frequency of 20 Hz, enzyme activity decreased by 21.0 ± 13.5% at the end of the 3rd hour of the experiment, at 24 Hz by 62.1 ± 15.1%, at 28 Hz by 33.0 ± 14.5%. Vibration with a frequency of 32 Hz contributed to the greatest decrease in Na^+,K^+-ATPase activity. Inactivation of the enzyme was 87.5 ± 5.9% at the end of the 3rd hour of the experiment. Vibration in the selected frequency range with A = 0.9 ± 0.08 mm led to a decrease in the activity of Na^+,K^+-ATPase, however, the decrease in activity did not exceed 5–10% relative to the initial level (Fig. 3b). An increase in enzyme activity was observed at the beginning of the experiment under the action of vibration with frequencies of 28, 32 Hz, A = 0.90 ± 0.08 mm. Thus, in the case of vibration with frequencies of 20–28 Hz, A = 0.50 ± 0.04 mm and 8–32 Hz, A = 0.90 ± 0.08 mm, we record an inverse correlation between Na^+,K^+-ATPase activity and ATP content.

Under the action of vibration, the nature of the change in the activity of cytoplasmic 5'-nucleotidase (cN) had a radically opposite nature, compared to the experiment without the action of vibration (Fig. 4a, 4b). In the entire studied frequency range, an increase in the activity of the enzyme by 1.42–2.68 times relative to the initial level was observed until the end of the experiment.

Under the action of vibration with a frequency of 8–16 Hz, A = 0.50 ± 0.04 mm, the activity of AMPDA significantly increased towards the end of the experiment (Fig. 5a). With vibration exposure in the frequency range of 20–28 Hz, the activity of AMPDA reached maximum values in the time interval of 60–80 min (an increase in activity by 1.56 ± 0.20 times when acting at a frequency of 20 Hz, 1.40 ± 0.12 times – 24 Hz, 1.85 ± 0.05 times – 28 Hz) and towards the end of the experiment a tendency to decrease in activity was observed. With vibration exposure at a frequency of 32 Hz, the increase in AMPDA activity (40–45%) was recorded only at the end of the experiment.

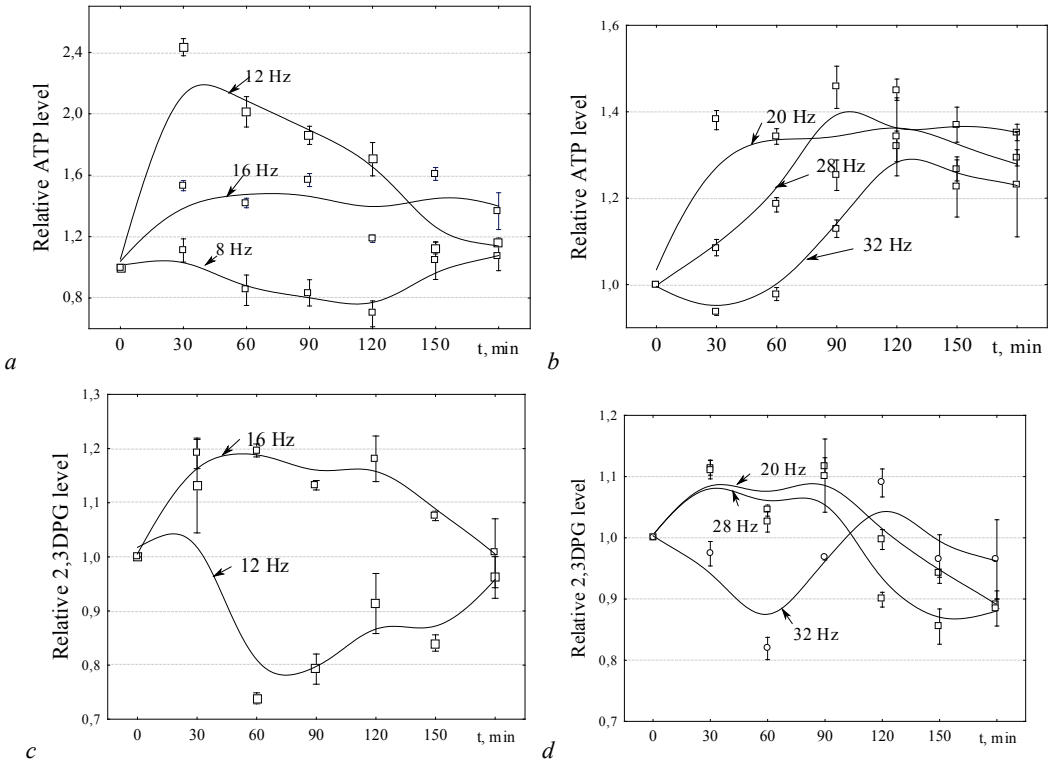

Рис. 2. ATP (*a*, *b*) and 2,3-BPG (*c*, *d*) content in erythrocytes relative to the initial level:
vibration impact parameters: frequency 8–32 Hz, A = 0.90 ± 0.08 mm; each point is represented as x ± SE, n = 3

Under the influence of vibration in the frequency range of 8–16 Hz, A = 0.90 ± 0.08 mm, the activity of AMPDA (Fig. 5b) increased during the third hour and reached a level exceeding the initial level by 1.4–1.7 times. In the frequency range of 20–28 Hz, the activity of AMPDA increased during the first 40 minutes (by 1.95 ± 0.12 and 1.50 ± 0.08 times under the influence of vibration with a frequency of 20 and 28 Hz, respectively). Further exposure led to a decrease in enzyme activity to the initial level. Under the action of vibration with a frequency of 32 Hz, the activity of AMPDA increased smoothly throughout the experiment (the maximum increase in activity was 55.6 ± 19.0% after 2 hours of vibration).

Changes in the activity of ecto-5'-nucleotidase (eN) under the conditions of vibration exposure are shown in Figure 6. In the frequency range of 8–20 Hz, A = 0.50 ± 0.04 mm, the activity of eN changed slightly. An increase in eN activity by 1.41–1.63 was observed under the action of vibration with frequencies of 24–32 Hz, A = 0.50 ± 0.04 mm during 2 hours of exposure and a tendency to decrease towards the end of the experiment (Fig. 6a). Under the action of vibration with A = 0.90 ± 0.08 mm in the frequency range of 8–16 Hz, the activity of eN increased. The greatest increase in activity was observed in experiments with a frequency of 8 Hz. No significant changes in eN activity were observed in the frequency range of 20–32 Hz (Fig. 6b).

Discussion

ATP is known not only as an energy carrier, but also as an important signaling molecule in many physiological processes (Yeung et al., 2018; Zhang et al., 2018; McMahon et al., 2021). ATP is a marker of functional activity and cell integrity (Ataullakhanov & Vitvitsky, 2002; Xu et al., 2019) and is of key importance in the energy-dependent maintenance of ionic and structural homeostasis of erythrocytes. This metabolite is a key factor in the local control loop of oxygen delivery. The ability of erythrocytes to deliver O_2 is regulated by many factors, including 2,3-BPG and ATP, which reduce the affinity of hemoglobin for O_2 and thus promote its release from hemoglobin (Peng et al., 2019; McMahon et al., 2021).

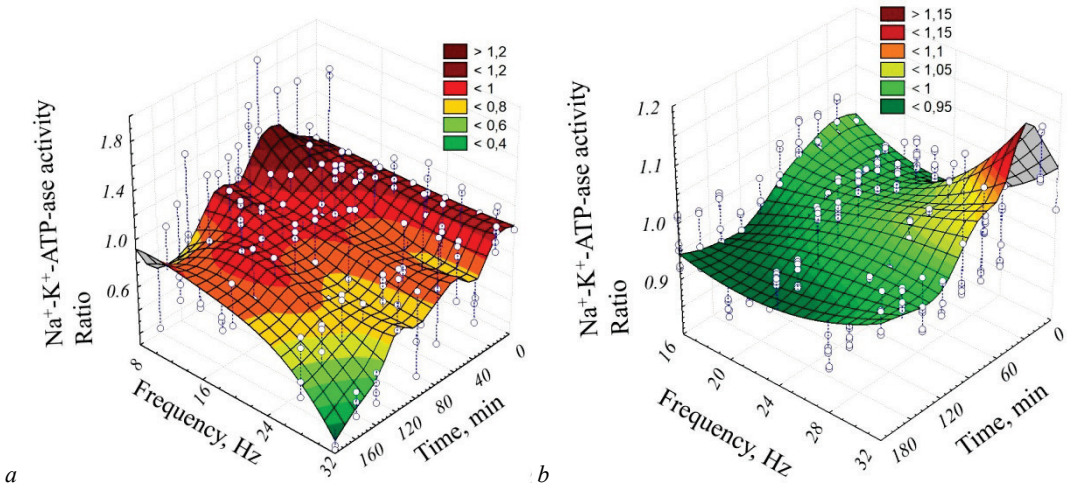

Fig. 3. Erythrocytes Na^+K^+-ATPase activity under the conditions of vibration exposure for 3 hours
in the frequency range of 8–32 Hz with amplitudes of 0.50 ± 0.04 mm (*a*) and 0.90 ± 0.08 (b): N = 250, n = 3

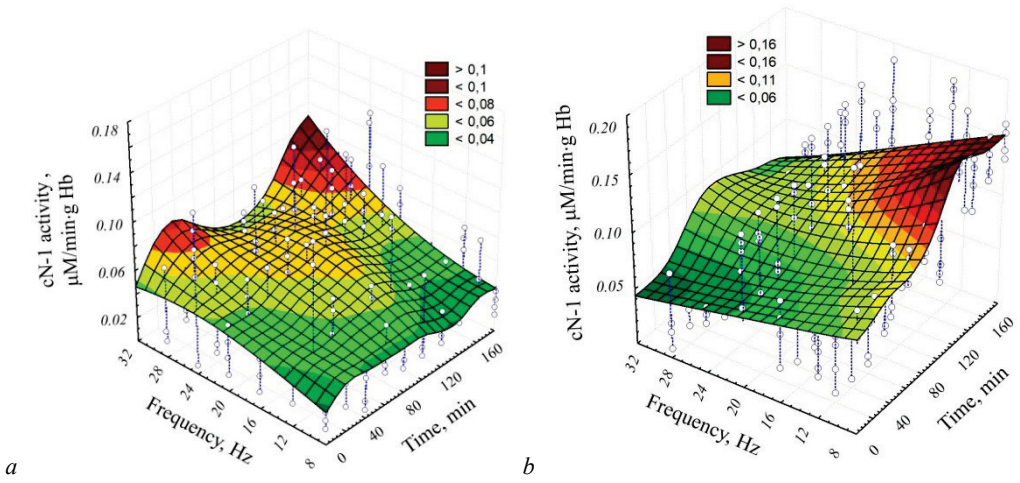

Fig. 4. Erythrocytes cN-1 activity under the conditions of vibration exposure for 3 hours in the frequency range of 8–32 Hz with amplitudes of 0.50 ± 0.04 mm (*a*) and 0.90 ± 0.08 (*b*): N = 250, n = 3

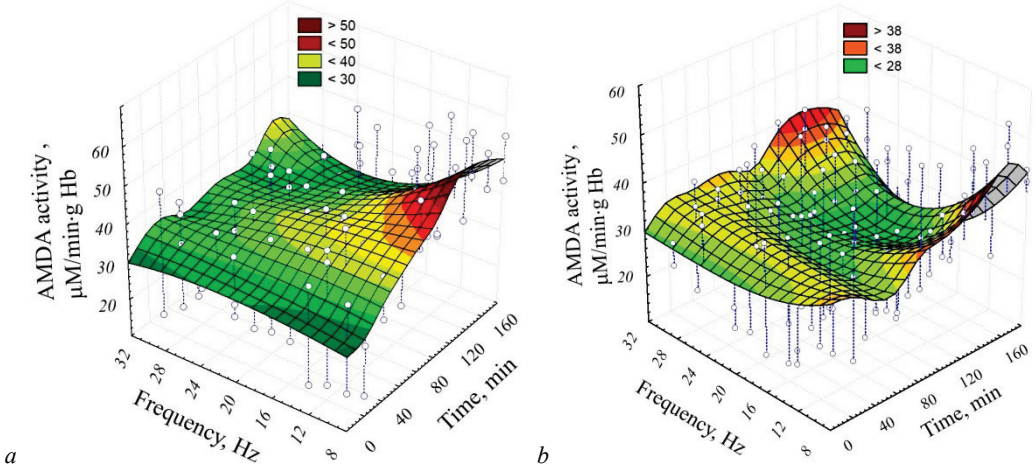

Fig. 5. Erythrocytes AMDA activity under the conditions of vibration exposure for 3 hours in the frequency range of 8–32 Hz with amplitudes of 0.50 ± 0.04 mm (*a*) and 0.90 ± 0.08 (*b*): N = 180, n = 3

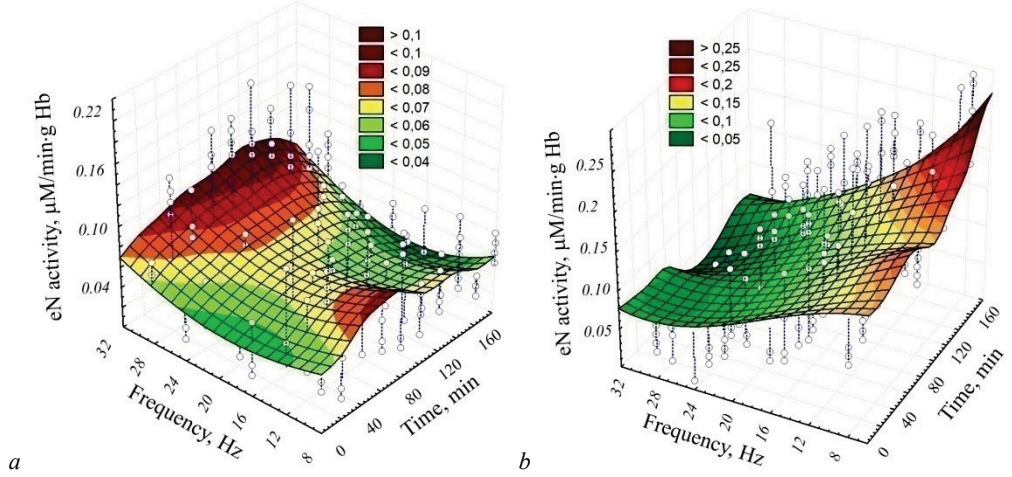

Fig. 6. Erythrocytes eN activity under the conditions of vibration exposure for 3 hours in the frequency range of 8–32 Hz with amplitudes of 0.50 ± 0.04 mm (*a*) and 0.90 ± 0.08 (*b*): N = 250, n = 3

The level of ATP in erythrocytes, like other purine nucleotides, is determined by the balance between its synthesis and the needs of the cell (Ataullakhanov & Vitvitsky, 2002). In the experiments, the incubation medium did not contain glucose, so the formation of de novo ATP was inhibited. In this regard, during the incubation of cells, we observed a gradual depletion of cells by ATP. Under the conditions of vibration in the frequency range of 20–28 Hz, A = 0.50 ± 0.04 mm, the ATP content was restored to the initial level, and under conditions of more intensive exposure (32 Hz, A = 0.50 ± 0.04 mm, 16–32 Hz, A = 0.90 ± 0.08 mm), the ATP content increased already after 20 min of exposure and remained at a certain level, or slightly decreased (Fig. 1, 2). Such changes are possible under the conditions of activation of certain mechanisms aimed at energy conservation and survival. This is indicated by a decrease in Na^+,K^+-ATPase activity in the entire frequency and amplitude range (Fig. 3). If in the experiments without vibration, the activity of Na^+,K^+-ATPase correlated with the ATP content in the cell, then under the conditions of vibra-

tion, the activity of the enzyme decreased and the degree of inactivation depended on the intensity of exposure. Thus, in the frequency interval of 24–32 Hz A = 0.9 ± 0.08 mm, we recorded a decrease in Na^+,K^+-ATPase activity already at the beginning of the experiment, despite the fact that the ATP content in the cell was increasing.

As is known, 2,3-BPG and ATP are negative allosteric regulators of hemoglobin oxygen affinity (D'-Alessandro & Xia, 2020; Chen et al., 2023). An increase in the content of 2,3-BPG and ATP indicates a state of hypoxia, which increases under conditions of increased intensity of vibration. Earlier, we showed that the content of deoxyhemoglobin in the cytoplasmic and membrane-bound fractions increases precisely under the conditions of vibration effect of 24–32 Hz A = 0.90 ± 0.08 mm (Dotsenko et al., 2021).

AMP content was assessed by the activities of 5'-nucleotidase (cN-1A) and AMP-deaminase (AMPDA) enzymes, which catalyze AMP. As previously shown (Ataullakhanov & Vitvitsky, 2002), high concentrations of AMP activate AMPDA, which has high activity. Involvement of this enzyme in the deamination of AMP allows you to quickly stabilize the energy charge, but the adenylate pool will be reduced. Presumably, this happens with simple incubation of erythrocytes in a medium without glucose and under the action of vibration in the range of 8–16 Hz, A = 0.50 ± 0.04 mm. At low concentrations of AMP, cN-1A is active, but its activity is two orders of magnitude lower than AMPDA (Ataullakhanov & Vitvitsky, 2002; Davis et al., 2020). Switching to the 5'-nucleotidase pathway of AMP degradation and reducing the rate of catabolism allows stabilization of the adenylate pool. Under the influence of vibration, the activities of cN-1A and AMPDA increased over time (Fig. 4, 5). However, the contribution of the deaminase pathway was insignificant. Under the conditions of vibrations and the frequency interval of 24–32 Hz A = 0.90 ± 0.08 mm, the deaminase pathway dominates, which indicates an increase in the content of AMP in the cells.

The conditions recorded experimentally are favorable for the activation of AMP-kinase (AMPK), which is an energy-sensitive enzyme and is activated by a sharp increase in the [AMP]/[ATP] ratio in the cell (Fujii et al., 2006; Kiger et al., 2021). It is the involvement of this enzyme that stabilizes the energy charge (Oakhill et al., 2012; Dotsenko & Troshchynskaya, 2014). AMPK is activated by a mechanism that involves allosteric modification and phosphorylation (Steinberg & Carling, 2019; Dengler, 2020). When cells experience a decrease in ATP, AMPK turns off pathways for ATP consumption and turns on alternative pathways for ATP regeneration. One hypothesis is that a decrease in intracellular ATP and a concomitant increase in AMP increase the AMP/ATP ratio (Fujii et al., 2006), causing conformational changes in the AMPK complex and phosphorylation of the α-subunit of the enzyme by one or more upstream kinases (Oakhill et al., 2012; Dengler, 2020). Thr172 is the main regulatory site of phosphorylation in the α-subunit of the enzyme, and its phosphorylation is important for catalytic activity. This signaling pathway starts with adenosine receptors (ADORA2B).

Activation of the erythrocyte-specific ADORA2B–AMPK–BPGM signaling axis contributes to hypoxic metabolic reprogramming of erythrocytes (Nemkov et al., 2021), the task of which is to enhance glycolysis, increase the content of 2,3-BPG and ATP, which contribute to the unloading of HbO_2 to counteract hypoxia. For this, erythrocytes must release a certain amount of ATP. In the extracellular environment, ATP is rapidly hydrolyzed by eATPase and two ectonucleotidases (triphosphate diphosphohydrolase (CD39) and monophosphohydrolase (CD73, eN)) to adenosine (Schneider et al., 2019). Adenosine can be imported back into erythrocytes by equilibrium nucleotide transporters (ENT1) or stimulate adenosine receptors. ADORA2b transduces an intracellular signaling pathway that activates protein kinase A and AMP-dependent kinase (AMPK). Both kinases promote metabolic reprogramming through phosphorylation-mediated activation of bisphosphoglycerate kinase (Qiang et al., 2021; D'Alessandro et al., 2023).

In recent years, systematic in vitro experimental studies have shown that the release of ATP by erythrocytes is quite sensitive to low oxygen levels, shear stress and rather high shape deformation (Zhang et al., 2018; Ferguson et al., 2021; Gou et al., 2021; Kirby et al., 2021; McMahon et al., 2021). However, according to the experimental data of the intracellular ATP content obtained in the work, it is difficult to say whether this happened. What data obtained in the work may indicate the activation of the ADORA2B–AMPK–BPGM signaling axis due to the release of ATP?

First, this is indicated by the nature of the change in AMP catabolism enzymes. We drew attention to the fact that in vibration conditions the activity of AMPDA increased at the beginning of the experiment and then mostly decreased (Fig. 5). The exception was the behavior of this enzyme in the case of vibration with a frequency of 8 Hz and with a very high intensity (24–32 Hz, A = 0.90 ± 0.08 mm). Chen et al. (2023) showed that adenosine fed back through ENT1 is further metabolized to AMP but not to IMP due to inhibition of AMPDA. It is not clear what exactly inhibits AMPDA, but it makes it possible to increase the level of AMP and activate AMPK. It was shown Chen et al. (2023), ablation of AMPDA3 (the AMPDA isoform expressed in erythrocytes) promotes higher ATP levels in mouse erythrocytes. Chen et al. (2023) provide evidence that AMPDA functions as a "hypoxic erythrocyte adenosine sensor". By controlling intracellular purine metabolism, AMPDA contributes to AMPK-BPGM-mediated metabolic reprogramming, induction of 2,3-BPG production, O_2 delivery, and antioxidant defense.

Secondly, to confirm the existence of a reverse activation pathway through ADORA2B, we examined changes in ectonucleotidase activity (eN, CD73). This enzyme is membrane-associated and decomposes AMP to adenosine in the extracellular environment. We established that the activity of eN increased under the conditions of vibration in the frequency range of 8–32 Hz, A = 0.50 ± 0.04 mm, which indicates at least the presence of the substrate in the extracellular environment (Fig. 6). However, under the conditions of exposure with high intensity (24–32 Hz, A = 0.90 ± 0.08 mm), the activity of eN practically did not change (Fig. 6b), which seems to contradict all the conclusions made above. However, it is known that CD73 can disappear from the cell surface after activation into a soluble state (sCD73). sCD73 activity has been detected in body fluids of patients with inflammation and cancer (Schneider et al., 2019) along with increased levels of adenosine (Liu et al., 2018). The membrane-bound form of 5'-nucleotidase (eN) binds to the lipid bilayer through a glycosylphosphatidylinositol (GPI-anchor) and this interaction significantly affects the activity of the enzyme (Dotsenko et al., 2014; Liu et al., 2018; Schneider et al., 2019). Possible mechanisms of release from the cell membrane include cleavage by metalloproteinases or cellular phospholipases. It is known that ADORA2B transmits a signal through both the G's and G'q subunits of the trimeric G-protein (Chen et al., 2014), so it is possible that the transition of CD73 to the soluble state occurs through the activation of the phospholipase pathway by adenosine.

Importantly, like many other GPI-anchored proteins, CD73 at the cell membrane is preferentially localized to detergent-resistant domains or lipid rafts, which often promote the formation of extracellular vesicles (EVs). Thus, according to our assumptions, at a high intensity of vibration, conditions of acute hypoxia probably occur, which leads to the release of CD73 from the membrane and an increase in its activity in the extracellular environment. However, this assumption requires experimental verification.

Conclusions

It was shown that under certain conditions of vibration exposure (frequency interval 20–32 Hz, A = 0.50 ± 0.04 mm and 12–32 Hz, A = 0.50 ± 0.04 mm) erythrocytes use signaling and metabolic pathways aimed at increasing the content of ATP, 2,3-BPG and restoration of the energy charge of cells. One of these pathways is controlled by AMP-kinase (AMPK), which in turn is a participant in the signaling cascade that begins with adenosine receptors ADORA2B. AMPK turns off consumption pathways and turns on alternative pathways for ATP regeneration, activates 2,3-BPG formation mechanisms. These ways are aimed at overcoming the state of hypoxia. The participation of AMP catabolism enzymes in ATP recovery processes is shown.

References

Ataullakhanov, F. I., & Vitvitsky, V. M. (2002). What determines the intracellular ATP concentration. Bioscience Reports, 22(5–6), 501–511.

Bunkin, N. F., Ninham, B. W., Ignatiev, P. S., Kozlov, V. A., Shkirin, A. V., & Starosvetskij, A. V. (2011). Long-living nano-bubbles of dissolved gas in

aqueous solutions of salts and erythrocyte suspensions. Journal Biophotonics, 4(3), 150–164.
Chen, C., Xie, T., Zhang, Y., Wang, Y., Yu, F., Lin, L., Zhang, W., Brown, B. C., Zhang, X., Kellems, R. E., D'Alessandro, A., & Xia, Y. (2023). Erythrocyte ENT1-AMPD3 axis is an essential purinergic hypoxia sensor and energy regulator combating CKD in a mouse model. Journal of the American Society of Nephrology, 34(10), 1647–1671.
Chen, J. F., Lee, C. F., & Chern, Y. (2014). Adenosine receptor neurobiology: Overview. International Review of Neurobiology, 119, 1–49.
Cilek, N., Ugurel, E., Goksel, E., & Yalcin, O. (2023). Signaling mechanisms in red blood cells: A view through the protein phosphorylation and deformability. Journal of Cellular Physiology, 2023, 30958.
D'Alessandro, A., & Xia, Y. (2020). Erythrocyte adaptive metabolic reprogramming under physiological and pathological hypoxia. Current Opinion in Hematology, 27(3), 155–162.
D'Alessandro, A., Anastasiadi, A. T., Tzounakas, V. L., Nemkov, T., Reisz, J. A., Kriebardis, A. G., Zimring, J. C., Spitalnik, S. L., & Busch, M. P. (2023). Red blood cell metabolism *in vivo* and *in vitro*. Metabolites, 13(7), 793–813.
Davis, P. R., Miller, S. G., Verhoeven, N. A., Morgan, J. S., Tulis, D. A., Witczak, C. A., & Brault, J. J. (2020). Increased AMP deaminase activity decreases ATP content and slows protein degradation in cultured skeletal muscle. Metabolism: Clinical and Experimental, 108, 154257–154277.
Dengler, F. (2020). Activation of AMPK under hypoxia: Many roads leading to Rome. International Journal of Molecular Sciences, 21(7), 2428–2443.
Deryugina, A. V., Boyarinov, G. A., Simutis, I. S., Nikolskiy, V. O., Kuznetsov, A. B., & Efimova, T. S. (2018). Correction of meta-bolic indicators of erythrocytes and myocardium structure with ozonized red blood-cell. Cell and Tissue Biology, 12, 207–212.
Dotsenko, O. I., & Troshchynskaya, Y. A. (2014). Role of AMP catabolism enzymes in the energetic status of erythrocytes under conditions of glucose depletion. Biosystems Diversity, 22(1), 46–52 (in Russian).
Dotsenko, O. I., Konyukhova, N. R., & Troshchynskaya, Y. A. (2013). Reguljacija strukturnogo stanu bilkiv cytoskeletu erytrocytiv pry dii' na nyh nyz'kochastotnoji vibraciji [Regulation of structural state of erythrocytes' cytoskeleton proteins at low-frequency vibration action]. Visnyk Donec'kogo Nacional'nogo Universytetu, Pryrodnychi Nauky, 1, 149–156 (in Ukrainian).
Dotsenko, O. I., Taradina, G. V., & Mischenko, A. M. (2021). Peroxidase activity of erythrocytes hemoglobin under action of low-frequency vibration. Studia Biologica, 15(4), 3–16.
Ellsworth, M. L., Ellis, C. G., & Sprague, R. S. (2016). Role of erythrocyte-released ATP in the regulation of microvascular oxygen supply in skeletal muscle. Acta Physiologica, 216(3), 265–276.
Ferguson, B. S., Neidert, L. E., Rogatzki, M. J., Lohse, K. R., Gladden, L. B., & Kluess, H. A. (2021). Red blood cell ATP release correlates with red blood cell hemolysis. American Journal of Physiology, Cell Physiology, 321(5), C761–C769.
Fujii, N., Jessen, N., & Goodyear, L. J. (2006). AMP-activated protein kinase and the regulation of glucose transport. American Journal of Physiology, Endocrinology and Metabolism, 291(5), E867–E877.
Gou, Z., Zhang, H., Abbasi, M., & Misbah, C. (2021). Red blood cells under flow show maximal ATP release for specific hematocrit. Biophysical Journal, 120(21), 4819–4831.
Grygorczyk, R., & Orlov, S. N. (2017). Effects of hypoxia on erythrocyte membrane properties-implications for intravascular hemolysis and purinergic control of blood flow. Frontiers in Physiology, 8, 1110.
Gudkov, S. V., Lyakhov, G. A., Pustovoy, V. I., & Shcherbakov, I. A. (2021). Vibration-vortex mechanism of radical-reaction activation in an aqueous solution: Physical analogies. Physics of Wave Phenomena, 29(2), 108–113.
Kirby, B. S., Sparks, M. A., Lazarowski, E. R., Lopez Domowicz, D. A., Zhu, H., & McMahon, T. J. (2021). Pannexin 1 channels control the hemodynamic response to hypoxia by regulating O_2-sensitive extracellular ATP in blood. American Journal of Physiology, Heart and Circulatory Physiology, 320(3), H1055–H1065.
Leal Denis, M. F., Alvarez, H. A., Lauri, N., Alvarez, C. L., Chara, O., & Schwarzbaum, P. J. (2016). Dynamic regulation of cell volume and extracellular ATP of human erythrocytes. PloS One, 11(6), e0158305.
Lew, V. L. (2023). The circulatory dynamics of human red blood cell homeostasis: Oxy-deoxy and PIEZO1-triggered changes. Biophysical Journal, 122(3), 484–495.
Liu, H., Adebiyi, M., Liu, R. R., Song, A., Manalo, J., Wen, Y. E., Wen, A. Q., Weng, T., Ko, J., Idowu, M., Kellems, R. E., Eltzschig, H. K., Blackburn, M. R., Juneja, H. S., & Xia, Y. (2018). Elevated ecto-5'-nucleotidase: A missing pathogenic factor and new therapeutic target for sickle cell disease. Blood Advances, 2(15), 1957–1968.
Liu, H., Zhang, Y., Wu, H., D'Alessandro, A., Yegutkin, G. G., Song, A., Sun, K., Li, J., Cheng, N. Y., Huang, A., Edward Wen, Y., Weng, T. T., Luo, F., Nemkov, T., Sun, H., Kellems, R. E., Karmouty-Quintana, H., Hansen, K. C., Zhao, B., Subudhi, A. W., … Xia, Y. (2016). Beneficial role of erythrocyte adenosine A2B receptor-mediated AMP-activated protein kinase activation in high-altitude hypoxia. Circulation, 134(5), 405–421.
Locovei, S., Bao, L., & Dahl, G. (2006). Pannexin 1 in erythrocytes: Function without a gap. Proceedings of the National Academy of Sciences of the United States of America, 103(20), 7655–7659.
Lushchak, V. I., Husak, V. V., & Storey, K. B. (2008). Regulation of AMP-deaminase activity from white muscle of common carp *Cyprinus carpio*. Comparative Biochemistry and Physiology, Part B, Biochemistry and Molecular Biology, 149(2), 362–369.
Marginedas-Freixa, I., Alvarez, C. L., Moras, M., Leal Denis, M. F., Hattab, C., Halle, F., Bihel, F., Mouro-Chanteloup, I., Lefevre, S. D., Le Van Kim, C., Schwarzbaum, P. J., & Ostuni, M. A. (2018). Human erythrocytes release ATP by a novel pathway involving VDAC oligomerization independent of pannexin-1. Scientific Reports, 8(1), 11384–11397.
McMahon, T. J., Darrow, C. C., Hoehn, B. A., & Zhu, H. (2021). Generation and export of red blood cell ATP in health and disease. Frontiers in Physiology, 12, 754638–754640.
Misiti, F., Carelli-Alinovi, C., & Rodio, A. (2022). ATP release from erythrocytes: A role of adenosine. Clinical Hemorheology and Microcirculation, 80(2), 61–71.
Montalbetti, N., Leal Denis, M. F., Pignataro, O. P., Kobatake, E., Lazarowski, E. R., & Schwarzbaum, P. J. (2011). Homeostasis of extracellular ATP in human erythrocytes. The Journal of Biological Chemistry, 286(44), 38397–38407.
Oakhill, J. S., Scott, J. W., & Kemp, B. E. (2012). AMPK functions as an adenylate charge-regulated protein kinase. Trends in Endocrinology and Metabolism, 23(3), 125–132.
Peng, Z., Luo, R., Xie, T., Zhang, W., Liu, H., Wang, W., Tao, L., Kellems, R. E., & Xia, Y. (2019). Erythrocyte adenosine A2B receptor-mediated AMPK activation: A missing component counteracting CKD by promoting oxygen delivery. Journal of the American Society of Nephrology, 30(8), 1413–1424.
Qiang, Q., Manalo, J. M., Sun, H., Zhang, Y., Song, A., Wen, A. Q., Wen, Y. E., Chen, C., Liu, H., Cui, Y., Nemkov, T., Reisz, J. A., Edwards Iii, G., Perreira, F. A., Kellems, R. E., Soto, C., D'Alessandro, A., & Xia, Y. (2021). Erythrocyte adenosine A2B receptor prevents cognitive and auditory dysfunction by promoting hypoxic and metabolic reprogramming. PLoS Biology, 19(6), e3001239.
Racine, M. L., & Dinenno, F. A. (2019). Reduced deformability contributes to impaired deoxygenation-induced ATP release from red blood cells of older adult humans. The Journal of Physiology, 597(17), 4503–4519.
Rozhkovskij, I. V., & Kresiun, V. I. (1991). Aktivnost' markernykh fermentov i sostoianie lipidnogo matriksa membranéritrotsitov pri stresse i ego medikamenoznoj korrektsii [Activity of marker enzymes and status of the erythrocyte membrane lipid matrix in stress and during its correction using medications]. Ukrainskij Biokhimicheskij Zhurnal, 63(4), 74–80 (in Russian).
Schneider, E., Rissiek, A., Winzer, R., Puig, B., Rissiek, B., Haag, F., Mittrücker, H. W., Magnus, T., & Tolosa, E. (2019). Generation and function of non-cell-bound CD73 in inflammation. Frontiers in Immunology, 10, 1729–1737.
Song, A., Zhang, Y., Han, L., Yegutkin, G. G., Liu, H., Sun, K., D'Alessandro, A., Li, J., Karmouty-Quintana, H., Iriyama, T., Weng, T., Zhao, S., Wang, W., Wu, H., Nemkov, T., Subudhi, A. W., Jameson-Van Houten, S., Julian, C. G., Lovering, A. T., Hansen, K. C., … Xia, Y. (2017). Erythrocytes retain hypoxic adenosine response for faster acclimatization upon re-ascent. Nature Communications, 8, 14108–14121.
Sridharan, M., Bowles, E. A., Richards, J. P., Krantic, M., Davis, K. L., Dietrich, K. A., Stephenson, A. H., Ellsworth, M. L., & Sprague, R. S. (2012). Prostacyclin receptor-mediated ATP release from erythrocytes requires the voltage-dependent anion channel. American Journal of Physiology, Heart and Circulatory Physiology, 302(3), H553–H559.
Steinberg, G. R., & Carling, D. (2019). AMP-activated protein kinase: The current landscape for drug development. Nature Reviews, Drug Discovery, 18(7), 527–551.
Takahashi, M., Shirai, Y., & Sugawa, S. (2021). Free-radical generation from bulk nanobubbles in aqueous electrolyte solutions: ESR spin-trap observation of microbubble-treated water. Langmuir, 37(16), 5005–5011.
Ugurel, E., Goksel, E., Cilek, N., Kaga, E., & Yalcin, O. (2022). Proteomic analysis of the role of the adenylyl cyclase-cAMP pathway in red blood cell mechanical responses. Cells, 11(7), 1250–1269.
Vaisey, G., Banerjee, P., North, A. J., Haselwandter, C. A., & MacKinnon, R. (2022). Piezo1 as a force-through-membrane sensor in red blood cells. eLife, 11, e82621.
Xu, Z., Dou, W., Wang, C., & Sun, Y. (2019). Stiffness and ATP recovery of stored red blood cells in serum. Microsystems and Nanoengineering, 5, 51–59.
Yeung, P. K., Kolathuru, S. S., Mohammadizadeh, S., Akhoundi, F., & Linderfield, B. (2018). Adenosine 5'-triphosphate metabolism in red blood cells as a potential biomarker for post-exercise hypotension and a drug target for cardiovascular protection. Metabolites, 8(2), 30–43.
Zhang, H., Shen, Z., Hogan, B., Barakat, A. I., & Misbah, C. (2018). ATP release by red blood cells under flow: Model and simulations. Biophysical Journal, 115(11), 2218–2229.