

Vibration influence on the O₂-dependent processes activity in human erythrocytes

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The early signs of vibration effects on the human body are microcirculation and transcapillary metabolism disorders, accompanied by disruption of the supply to and utilization of oxygen in the tissues and organs. However, there are few experimental studies aimed at finding targets of vibration in cells and determining the action mechanism of vibration. In *in vitro* experiments, human erythrocytes in buffer solution were exposed to low-frequency vibration (frequency range 8–32 Hz, amplitudes 0.5–0.9 mm) for 3 hours. The dynamics of the accumulation of membrane-bound catalase and hemoglobin and the distribution of ligand hemoglobin in the membrane-bound fraction were studied as the indicators of functional activity of cells. The choice of these indicators is justified by the participation of catalase and hemoglobin in O₂-dependent cellular reactions as a part of protein complexes. Since pO₂ is a trigger of conformational transitions in the hemoglobin molecule, simultaneously with oxygen transport, hemoglobin signals to different metabolic systems about oxygen conditions in the environment. The studies revealed that in the conditions of vibration, the activity of membrane-associated catalase increased by 40–50% in the frequency range of 12–24 Hz (amplitude 0.5 ± 0.04 mm), by 20–30% in the amplitude of 0.9 mm, but after about 100–120 min exposure the enzyme activity decreased even below the control level. There was a dose-dependent accumulation of membrane-bound hemoglobin during exposure to vibration. In the membrane-bound fraction of hemoglobin, oxyhemoglobin had the highest content (60–80%), while the content of methemoglobin varied 5–20%. During vibrations in the frequency range 12–28 Hz, 0.5 mm, we recorded 10–30% increase in oxyhemoglobin. With increase in the vibration amplitude (0.9 mm) in the frequency range of 16–32 Hz, constant content of oxyhemoglobin was noted at the beginning of the experiment, which tended to decrease during the last exposure time. Frequency of 32 Hz caused increase in the deoxyhemoglobin content in the membrane-bound fraction. The content of methemoglobin (metHb) in erythrocytes significantly increased during exposure to the frequency range of 12–24 Hz, with the amplitude of 0.5 mm (1.3–2.4 times). During the exposure to frequencies of 28 and 32 Hz, we observed the transition of methemoglobin to hemichrome. The content of methemoglobin in the cells was lower and decreased at the end of the experiment when the vibration amplitude was 0.9 mm. In these experimental conditions, no increase in hemichrome content in the membrane-bound fraction was recorded. Therefore, the degree of binding of catalase and hemoglobin with the membrane of erythrocytes that were exposed to vibration and the changes in the content of ligand forms in the composition of membrane-bound hemoglobin are dose-dependent. Low-frequency vibration initiates O₂-dependent processes in erythrocytes. Targets of such an influence are nanobubbles of dissolved air (babstons), retained on the surface of erythrocytes due to Coulomb interactions, capable of coagulation and increase in size under the action of vibration. At first, the consequences of these processes are increase in oxygen content in the surface of erythrocytes, and then decrease as a result of degassing. Thus, increase in oxygen content on the surface initiates redox reactions, whereas decrease in oxygen content leads to reconstruction of metabolic processes oriented at overcoming hypoxia.

Keywords: oxidative stress; catalase; reactive oxygen species; molecular oxygen; nanobubbles; ligand forms of hemoglobin; membrane-bound hemoglobin.

Introduction

The early signs of vibration effects on the human body are disorders of microcirculation and transcapillary metabolism, which are accompanied by disruption of oxygen supply and utilization in the tissues and organs and tissue hypoxia (Dotsenko et al., 2010; Vorobeve & Shabanov, 2019; Vorobeve & Shabanov, 2020). According to several authors (Kiryakov et al., 2018; Krajnak, 2018; Potapova, 2020) vascular disorders in vibration disease are associated with structural and functional changes in erythrocyte membranes, increasing their permeability to potassium and calcium, destruction of cytoskeletal proteins and malonic dialdehyde formation during the inactivation of antioxidant protection enzymes. However, there are few experimental studies aimed at finding targets of vibration in cells and determining the mechanism of its action on cells. We have previously shown that external low-frequency oscillations alter the activity of oxygen-dependent and metabolic reactions in erythrocytes (Dotsenko & Mischenko, 2011; Dotsenko et al., 2014). The studies revealed that at frequencies of 20–32 Hz, the reaction leading to aggregation

of cellular proteins prevails. These processes have a stabilizing effect on the membrane and premembrane cytoskeleton and enhance acid resistance of cells (Dotsenko & Mischenko, 2011).

The ability of proteins to reversibly bind with membrane components is considered to be one of the oldest mechanisms of response to external influences (Chu et al., 2016; Kosmachevskaya et al., 2019; Rodríguez-Bolaños et al., 2020; Issaian et al., 2021). These processes provide rapid response of the cell to the action of stressors. Trigger mechanisms of the transition of proteins from free to bound state are considered the emergence of interactive secondary structures formed by the folding of native-expanded or partial denaturation of globular proteins. These secondary structures become centers of “native aggregation” (Matveev, 2010; Jaeken & Matveev, 2012; Matveev, 2019; Cornish et al., 2020; Rodríguez-Bolaños et al., 2020) and provide highly specific intra- and interprotein interactions. As a result, there are temporary structures necessary to ensure functional activity. Localization of the process of development of protein complexes next to the membrane provides accurate modulation of cellular signaling events through spatio-temporal regulation of signaling comple-

xes (Saarikangas & Barral, 2016; Yogurtcu & Johnson, 2018; Cornish et al., 2020).

In erythrocytes, an example of reversible binding is the O₂-dependent association of hemoglobin with band 3 protein (CDB3). This process regulates the formation of complex of glycolytic enzymes on the erythrocyte membrane when glucose levels change, switches metabolism between pentose phosphate pathway and glycolysis, affects the ankyrin and CDB3 interaction with simultaneous regulation of erythrocyte membrane stability and ATP release from erythrocytes, which is related to vasodilation (Puchulu-Campanella et al., 2013; Chu et al., 2016; Kosmachevskaya et al., 2019; Zheng et al., 2019; Issaian et al., 2021). Hemoglobin is a gas- and redox-sensory protein that perceives changes in the environment, generates information signals, transmits them to metabolic structures, thereby self-regulating intracellular metabolic processes (Kosmachevskaya et al., 2019).

Recent studies have shown that antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx) and peroxiredoxin (Prx2) are also in membrane-associated state at the time of cell activity (Bayer et al., 2016; Melo et al., 2019; Rocha et al., 2019). The binding of these cytosolic enzymes to the membrane is thought to be associated with the oxidative stress resistance. The studies revealed that the content of membrane-bound antioxidant protection enzymes is enhanced in the presence of hematological diseases associated with oxidative stress, such as hereditary spherocytosis (Rocha et al., 2019), beta-thalassemia, erythrocyte enzymopathy, blood storage (Melo et al., 2019; Rocha et al., 2019).

Therefore, we studied the effect of low-frequency vibration (frequency range 8–32 Hz, amplitudes 0.50 ± 0.04 and 0.90 ± 0.08 mm) on the state of human erythrocytes. As indicators of the functional activity of cells, we studied the dynamics of accumulation of membrane-bound catalase and hemoglobin, the distribution of ligand hemoglobin in the membrane-bound fraction. We selected these indicators because of the participation of catalase and hemoglobin in O₂-dependent cellular reactions as a part of protein complexes. Because pO₂ is a trigger of conformational transitions in the hemoglobin molecule, simultaneously with oxygen transport, hemoglobin signals to different metabolic systems about oxygen conditions (Kosmachevskaya & Topunov, 2019). We used the sensory properties of hemoglobin as a marker of erythrocyte oxygen supply in the conditions of vibration and discussed a new paradigm that, in our opinion, could be useful for understanding of the targets and mechanisms of vibration effects on biological structures.

Materials and methods

The protocol of the experimental part corresponds to the principles of biological ethics and was approved by the Local Ethics Committee of the Vasylii Stus Donetsk National University, Faculty of Chemistry, Biology and Biotechnology (Vinnitsa, Ukraine).

Peripheral blood of almost healthy donors of one sex and about the same age was used in the study. Erythrocytes were separated from plasma by centrifugation and rinsed three times with Na-phosphate buffer (0.015 M, pH 7.4) containing 0.15 M NaCl (buffer solution 1). In the obtained erythrocyte paste, the total hemoglobin content was determined by unified method using hemoglobin cyanide and the standard kit.

The resulting erythrocyte paste was introduced into the medium of the same buffer solution. The hemoglobin content in the test suspensions was at the level of 2.55 ± 0.18 mg/mL. The suspensions were subjected to low-frequency vibration for 3 hours in the frequency range of 8 to 32 Hz. The vibration frequency varied with step of 4 Hz. The vibration amplitude was maintained at 0.50 ± 0.04 and 0.90 ± 0.08 mm. The vibration was carried out using a shaker consisting sinusoidal low-frequency signal generator, an amplifier and a vibrator that oscillates in the vertical plane with the given frequency and amplitude. The experimental cuvette filled with the erythrocyte suspension was fixed vertically tightly on the movable part of the vibrator (in this case, mechanical vibrations are transmitted to the experimental cuvette with slight losses of power).

During the experiment, we determined the activity of membrane-bound catalase, the content of membrane-bound hemoglobin, the content of ligand forms of hemoglobin in the composition of the membrane-bound fraction. As the control, erythrocytes that were not exposed to

vibration in buffer solution 1 were used. Membrane-bound catalase activity was determined using complete erythrocytes according to the rate of utilization of hydrogen peroxide (H₂O₂). The amount of H₂O₂ was determined using a Fox reagent (Wolff & Dean, 1987). The activity of the enzyme was expressed as $\mu\text{mol/L}$ of substrate (H₂O₂), converted by the enzyme per min, per mg of hemoglobin (Hb) in the sample.

To study the content of membrane-bound hemoglobin and its ligand forms, the erythrocytes were precipitated by centrifugation of aliquot of the working suspension. Cell lysis were performed by adding 5 (7) mL of 0.01 M Na-K-phosphate buffer (pH 7.4) at $T = 4^\circ\text{C}$. The ghosts were precipitated by centrifugation (10 min, 3000 rpm) and washed with buffer 1 and dissolved in 0.5 (0.2) mL of 5% Triton X-100 solution (Ratanasopa et al., 2015; Rocha et al., 2019). The sample was kept for 5 min until complete clarification of the solution, then buffer 1 added.

The absorption spectra of membrane-bound erythrocyte hemoglobin was recorded in the wavelength range of 500–700 nm in 1 mm thick cuvettes. A solution containing 0.5 (0.2) mL of 5% Triton X-100 and buffer solution 1 was used as the reference solution for spectrophotometric measurements in the study of membrane-bound hemoglobin.

The total content of membrane-bound hemoglobin fractions was determined by absorption at the wavelength of 523 nm using extinction coefficient of 7,120 M/cm (Ratanasopa et al., 2015), as well as by the integrated intensity of the absorption band. Determining the content of ligand forms of hemoglobin was determined as described previously in (Altia et al., 2015). The content of each form was expressed as percentage of the total hemoglobin content in the membrane-bound fraction.

All experiments were performed in three to five replicates, and their results were analyzed in Statistica 8.0 (StatSoft Inc., USA). Experimental data was presented as $x \pm m$ (x is the average, m is the error). Three-dimensional scattering graphs were used to present the obtained experimental data and identify the relationships between the studied parameters. Based on the network of starting points, the surface was developed using the method of inversely weighted distances. The method is based on the presumption that the value of the z attribute at any point in space where no measurements have been made is the weighted average of the values at the measurement points adjacent to a certain radius or window around that point.

Results

The changes in the activity of membrane-bound catalase in erythrocytes exposed to vibration (Fig. 1) show that changes in activity significantly depend on the amplitude of vibration. During vibration with amplitude of 0.50 ± 0.04 mm (Fig. 1a), we can point out the following features of changes in catalase activity. Exposure to 8 Hz frequency caused increase in the activity of the enzyme at the beginning of the experiment and decrease in catalase after 75 min of the exposure ($54.5 \pm 11.0\%$). In the frequency range of 12–24 Hz, the increase in enzyme activity was dose-dependent and was $57.7 \pm 20.6\%$, $16\text{ Hz} - 45.0 \pm 14.0\%$, $24\text{ Hz} - 36.4 \pm 16.4\%$ after 3 hours exposure to 12 Hz frequency. As the vibration frequency increased, the time interval from which the increase in enzyme activity was observed widened. In the frequency ranges of 28 and 32 Hz, the activity of the membrane-bound enzyme remained at the control level throughout the experiment (Fig. 1a).

When exposed to the amplitude of 0.90 ± 0.08 mm, increase in catalase activity was recorded only at 8 Hz frequency vibration (enzyme activity during the first 90 min of the experiment was at the control level and then increased by $28.6 \pm 9.1\%$). Frequency range 12–32 Hz led to 20–35% increase in enzyme activity during 90–120 min of the experiment with subsequent decrease in its activity (Fig. 1b). The change in the content of membrane-bound hemoglobin in erythrocytes in the conditions of low-frequency vibration with the amplitude of 0.50 ± 0.04 mm was weakly dependent on the frequency of vibration and dose-dependent (Fig. 2a). Subject to the action of vibration with 8 Hz frequency, membrane-bound hemoglobin rapidly accumulated in erythrocytes and by the end of the experiment its content doubled. Vibration in the frequency range of 12–32 Hz also led to a gradual increase in the content of membrane-bound hemoglobin. After 3 hours of the exposure, we recorded increase in the content of membrane-bound hemoglobin in erythrocytes by $61.6 \pm 7.7\%$

(16 Hz), by $47.2 \pm 12.2\%$ (20 Hz), by $77.4 \pm 13.2\%$ (24 Hz), by $48.5 \pm 18.2\%$ (28 Hz) and $47.1 \pm 11.9\%$ (32 Hz, Fig. 2a).

The increase in the content of membrane-bound hemoglobin exposed to the action of vibration with the amplitude of 0.90 ± 0.08 mm had a different pattern. No increase in membrane-bound hemoglobin was observed at 8 and 12 Hz. When exposed to the frequency of 16 Hz, increase in membrane-bound hemoglobin was observed only after one hour of the experiment, which by the end of the experiment was $36.2 \pm 19.1\%$, 20

Hz – $16.4 \pm 10.6\%$, 24 Hz – $45.4 \pm 2.4\%$. Exposure to frequency of 32 Hz (Fig. 2b) caused gradual increase in the content of membrane-bound hemoglobin throughout the time period, equaling $57.0 \pm 9.9\%$ at the end of the experiment.

The results showed that at the vibration amplitude of 0.50 ± 0.04 mm, the accumulation of membrane-bound hemoglobin in erythrocytes was higher than in cells exposed to vibration with the amplitude of 0.90 ± 0.08 mm.

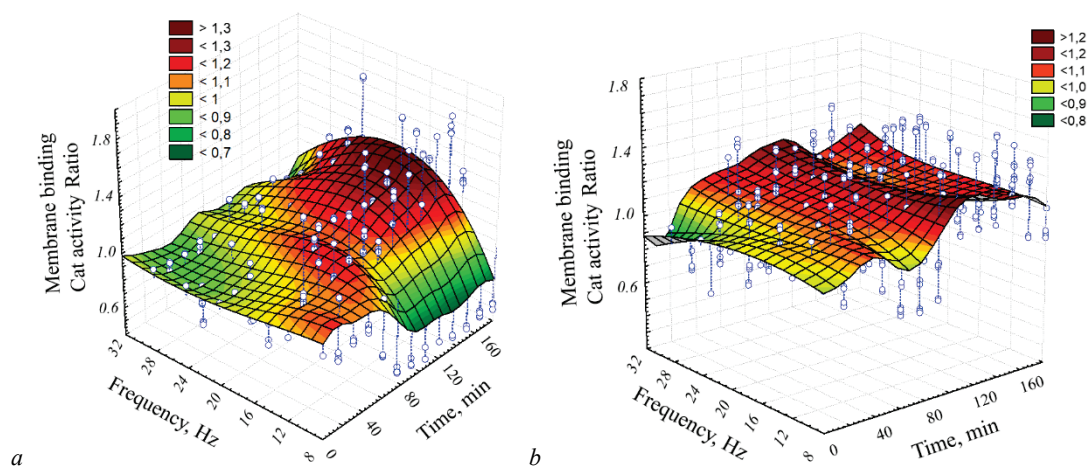


Fig. 1. The activity of membrane-bound erythrocyte catalase in the conditions of vibration in the frequency range of 8–32 Hz with amplitudes of 0.50 ± 0.04 mm (a) and 0.90 ± 0.08 (b) depending on the incubation time of cells: $N = 350$, $n = 5$

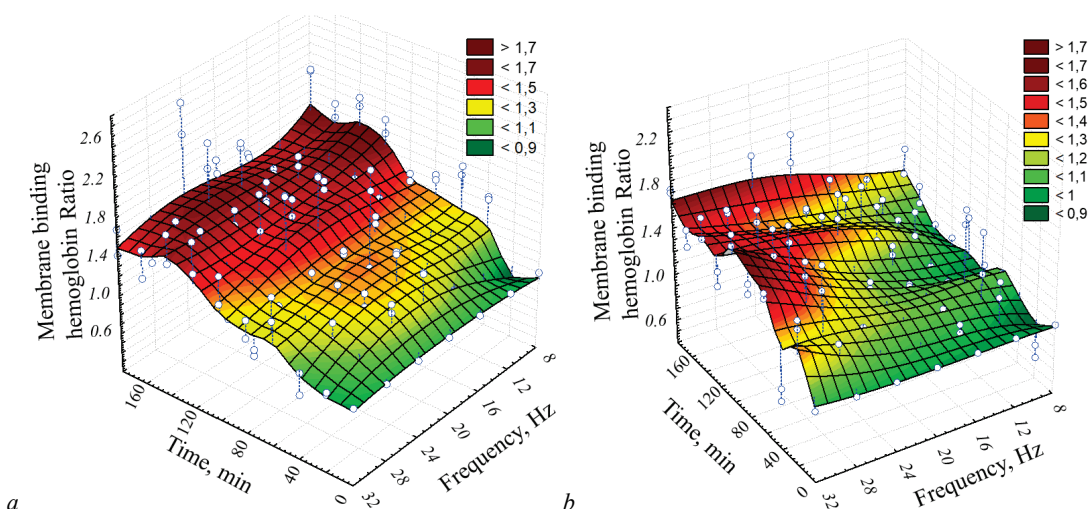


Fig. 2. The change in the content of membrane-bound hemoglobin of erythrocytes in the conditions of vibration in the frequency range 8–32 Hz with amplitudes of 0.50 ± 0.04 mm (a) and 0.90 ± 0.08 (b) depending on the incubation time of the cells; $N = 180$, $n = 3$

We analyzed the content of ligand forms of hemoglobin in the membrane-bound fraction. Figure 3 analyzes changes in oxyhemoglobin and Figure 4 – in methemoglobin. The nature of the change in oxyhemoglobin during vibration with the amplitude of 0.50 ± 0.04 mm was oscillating – it increased during the first hour of exposure and at the end of the experiment (Fig. 3a). During the vibration with 8 Hz frequency, the average increase in oxyhemoglobin was $25.8 \pm 7.7\%$. In the frequency range of 12–20 Hz, we observed $6.7 \pm 1.6\%$ increase in the amount of oxyhemoglobin in the membrane-bound fraction. The greatest increase in the content of oxyhemoglobin was recorded during the exposure to frequencies of 24 and 28 Hz (up to 30%). The frequency equaling 32 Hz caused no increase in oxyhemoglobin compared to the initial level.

Vibration with the amplitude of 0.90 ± 0.08 mm increased the content of oxyhemoglobin only at 8 Hz frequency. Vibration at 12 Hz frequency caused no changes in the content of oxyhemoglobin compared to the control. In the frequency range of 16–32 Hz, the content of oxyhemoglobin in the membrane-bound fraction decreased over time. The decrease in oxyhemoglobin was: $26.6 \pm 4.6\%$ – 16 Hz, $20.6 \pm 5.3\%$ – 20 Hz, $23.9 \pm 8.5\%$ – 24 Hz, $25.1 \pm 7.9\%$ – 28 Hz, $24.4 \pm 7.5\%$ – 32 Hz (Fig. 3b). Vibration with the frequency of 8 Hz and the amplitude of 0.50 ± 0.04 mm

decreased the content of methemoglobin by $38.2 \pm 8.3\%$ (Fig. 4). Exposure to 12, 16 and 20 Hz frequencies led to increase in the content of methemoglobin in the membrane-bound fraction by 1.3, 3.0 and 2.5 times, respectively (Fig. 4a). Exposure to the frequencies of 24–32 Hz led to decrease in the content of methemoglobin, which gradually turned into hemichrome. Under the action of vibration with the frequency of 32 Hz, the content of hemichrome in the cells increased by 2.5 times compared with the initial level.

Vibration with the amplitude of 0.90 ± 0.08 mm (Fig. 4b) in the frequency range of 8–32 Hz caused 1.5–2.0-fold increase in the content of methemoglobin during 80–120 min of the experiment, and then we recorded a tendency towards its decrease. Hemichrome did not accumulate.

Discussion

Erythrocytes are an example of a well-developed mechanism of adaptive response which involves proteins and membrane structures (Kosmachevskaya et al., 2019). Aggregation usually begins in response to cellular demand and may include: the formation of binding sites with ions, molecules or solutes, increased enzyme activity, channel formation and

assembly of supramolecular structures (Matveev, 2010; Jaeken & Matveev, 2012; Matveev, 2019; Cornish et al., 2020, Rodríguez-Bolaños et al., 2020). Thus, under certain circumstances, peptides rearrange to adapt to changes, initiating the process of reversible or irreversible aggregation, which takes place in certain pathological conditions. Erythrocytes are

characterized by the assembly of membrane multienzyme complexes. Organization of enzymes, which have common metabolites or coenzymes, in the metabolon provides effective advancement of intermediates along the conveyor of active centers, thus providing micro-compartmentalization of the metabolic process.

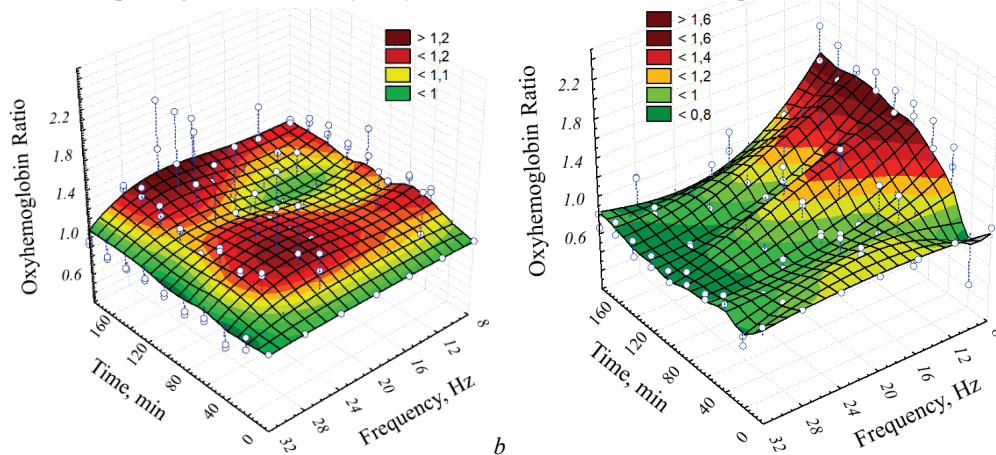


Fig. 3. The change of oxyhemoglobin content in the membrane-bound fraction of hemoglobin exposed to vibration in the frequency range of 8–32 Hz with amplitudes of 0.50 ± 0.04 mm (a) and 0.90 ± 0.08 (b) depending on the incubation time of cells: $N = 180$, $n = 3$

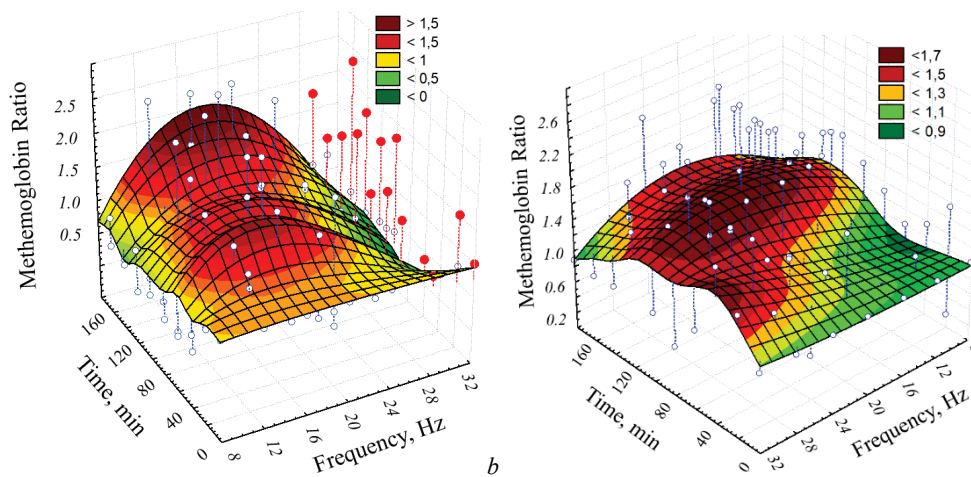


Fig. 4. The change in the content of methemoglobin in the membrane-bound fraction of hemoglobin exposed to vibration in the frequency range 8–32 Hz with amplitudes of 0.50 ± 0.04 mm (a) and 0.90 ± 0.08 (b) depending on the incubation time of cells: the red dots show the hemichrome content; $N = 180$, $n = 5$

Diatomic oxygen (O_2) is the most common signaling molecule that regulates the assembly of multienzyme complexes to control the properties of erythrocytes. It is well known that fluctuations in O_2 levels are accompanied by changes in the association of glycolytic enzymes with binding sites of protein 3, allowing glucose to be redirected from glycolytic to pentose phosphate pathway and vice versa (Puchulu-Campanella et al., 2013; Chu et al., 2016; Sidorenko et al., 2018; Issaian et al., 2021). At low levels of O_2 , the complex rapidly dissociates and releases glycolytic enzymes into the cytosol, resulting in increased glycolysis and ATP formation. Elevated O_2 levels promote the shunting of glucose to the pentose phosphate pathway, which results in the production of reduced NADPH, a metabolite that protects erythrocytes against oxidative stress. Increased O_2 levels enhance the interaction between band 3 protein and ankyrin, thereby stabilizing the erythrocyte membrane. At the same time, hypoxia leads to rupture of this important bridge and thus increases the deforming ability of cells (Stefanovic et al., 2013; Chu et al., 2016). Finally, deoxygenation promotes the release of ATP from erythrocytes, leading to activation of P2Y receptors on endothelial cells and, as a consequence, vasodilation, which facilitates blood flow from hypoxic tissues back to the lungs (Chu et al., 2016). The interaction between CBD3 and hemoglobin was found to be O_2 -regulated molecular switch that can generate a signal and transmit it to metabolic structures (Chu et al., 2016; Kosmachevskaya et al., 2019). The binding of hemoglobin and other enzymes to CBD3 is reversible and depends on O_2 pressure (Welbourn et al., 2017; Zhou et al., 2019).

To study the targets of low-frequency vibration, simple systems are used, namely solutions of proteins or cell suspensions. A large amount of evidence has been collected, suggesting that mechanical stress can affect the properties of biomolecules and cells (Dotsenko & Mischenko, 2011; Dotsenko et al., 2014; Bunkin et al., 2020). Erythrocytes exposed to vibration were kept in buffer solution saturated with O_2 (Chwastowski et al., 2020). O_2 itself, along with other gases, is found in bubbles with the diameter of less than a micron – nanobubbles (Bunkin et al., 2011; Shatalov et al., 2012; Li & Bunkin, 2019). The lifetime of micron bubbles is 10^{-2} s, but their stability is increased in saline solutions due to the adsorption of ions on the surface of the bubble (Bunkin et al., 2016; Uchida et al., 2016). The term “babston” (bubble stabilized by ions) was introduced to denote stable nanobubbles that spontaneously occur under normal conditions in liquids saturated with dissolved gas and contain an ionic component (Bunkin et al., 2011). The existence of a gas-liquid interface plays an important role in the formation of nanobubbles, and fluctuations in water properties may be associated with chaotic fluctuations in the concentration of air dissolved in water and the number of microbubbles (Shatalov et al., 2012). Babstons tend to coagulate, forming babston clusters of long term microhomogeneities in aqueous ionic solutions (Bunkin et al., 2011; Bunkin et al., 2016; Li & Bunkin, 2019).

It turned out that the concentration of nanobubbles increases significantly after vibration treatment of water or solution and is determined by the frequency and time of vibration. In addition, the average size of nanobubbles gradually increases according to the duration of vibration (Fang

et al., 2020). The growth of bubbles is due to cavitation, which releases surface energy (Shatalov et al., 2012). It has been experimentally proven that under the action of vibration, nanobubbles are a source of reactive oxygen species (Gudkov et al., 2020; Gudkov et al., 2021; Takahashi et al., 2021). Gudkov et al. (2020) showed that the relative rate of formation of hydrogen peroxide in water saturated with atmospheric gases is approximately 0.8 nM/min when exposed to the frequency of 30 Hz. The studies revealed that the rate of generation of hydrogen peroxide increases with increasing frequency of mechanical loads proportionally to f^2 . The consequence of further growth of microbubbles is degassing of liquid as a result of the multi-fold increased force of Archimedes, which pushes the microbubbles to the surface of the solution (Shatalov et al., 2012). The presence of degassing was confirmed by several studies (Shatalov, 2012; Gudkov et al., 2021).

The negative charge of the erythrocyte surface attracts positively charged “babstones” to its surface. The existence of bubbles or their clusters on the surface of the erythrocyte was confirmed by combining the techniques of laser scatterometry and modulation interference microscopy. (Bunkin et al., 2011). The role of bubble clusters on the surface of erythrocytes has not been studied. Oxygen of bubbles is considered to be involved in the processes of oxygenation-deoxygenation with the participation of hemoglobin. It has been experimentally shown that degassing of blood plasma increases ESR (erythrocyte sedimentation rate) (Shatalov, 2012).

A certain amount of catalase is always bound to erythrocyte membrane components (Bayer et al., 2016; Melo et al., 2019; Rocha et al., 2019). Its content depends on the degree of oxidative stress and the appearance of hydrogen peroxide (Melo et al., 2019; Rocha et al., 2019). The increase in catalase activity by 40–50% (Fig. 1a) during vibration with the frequency range of 12–24 Hz, with the amplitude of 0.50 ± 0.04 mm may be associated with the formation of H_2O_2 in the buffer solution. H_2O_2 inactivation requires more catalase near the cell surface. There may be another explanation. Increase in the number and size of nanobubbles on the surface of the erythrocyte lead to increase in oxygen concentration. Oxygenation of hemoglobin enhances the processes of its autooxidation, in which a radical is formed. SOD1 catalyzes the dismutation reaction with the formation of H_2O_2 . Catalase is needed to break down H_2O_2 and protect hemoglobin against oxidation. Binding of SOD1 with the membrane was demonstrated in (Sidorenko et al., 2018). As a result, a temporary protein complex assembles, the action of which is aimed at inactivating ROS. The second version seems more likely to us, for the amount of hydrogen peroxide recorded in aqueous solutions under the action of vibration was insignificant.

We should note that vibration with the amplitude of 0.90 ± 0.08 mm led to lower increase in the activity of membrane-bound catalase (20–30%) and after about 100–120 min the activity of the enzyme decreased even to a lower level than the control. Increase in the amplitude led to increase in vibration acceleration and its intensity, resulting in rapid increase in bubbles and their ascent to the surface. As a result of degassing, the level of oxygen in the erythrocyte surface decreased, thus the amount of bound catalase decreases.

If the presence of a certain amount of catalase in the membrane protein complex is a proven fact, its location is unknown. Accurate mapping of the interfaces between AE1 and number of enzymes (Issaian et al., 2021) revealed that peroxiredoxin and catalase can bind to the N-terminal domain of CBD3. Based on the obtained experimental data, Storozhuk et al. (2000) suggested that molecular oxygen, which is released from hydrogen peroxide under the influence of catalase, accelerates as a result of the reaction and starts the process of hemoglobin oxygenation. Only then does hemoglobin begin to interact with atmospheric O_2 . Although this hypothesis is not generally accepted, we believe that it is noteworthy. The data we obtained indicate that the binding of catalase to the membrane is also an O_2 -dependent process; the level of bound catalase decreased in the case of hypoxic state. The physiological role of catalase binding is to protect hemoglobin against oxidation.

In the recent years, special attention has been paid to the participation of hemoglobin in the transmission of signals inside the erythrocyte. According to the study, vibration with the amplitude of 0.50 ± 0.04 mm led to accumulation of membrane-bound hemoglobin. This process was dose-dependent, but not frequency-dependent (Fig. 2a). During vibration with

the amplitude of 0.90 ± 0.08 mm, slightly lower accumulation of membrane-bound hemoglobin was observed, except for the experiment with the frequency of 32 Hz (Fig. 2b). The amount of bound hemoglobin is not an informative indicator, for several binding sites are known, including the binding of deoxyhemoglobin (deoxyHb) to the protein of band 3, hemoglobin binding to the cytoskeletal node formed by spectrin, ankyrin and protein of band 4.2, cytoskeletal proteins (Welbourn et al., 2017; Kosmachevskaya et al., 2019). Therefore, we further investigate the content of ligand forms in the composition of membrane-bound hemoglobin.

In the membrane-bound fraction of hemoglobin, oxyhemoglobin accounted for the largest share (60–80%), while the content of methemoglobin varied 5–20%. With exposure to vibration with the amplitude of 0.50 ± 0.04 mm, the amount of oxyhemoglobin in the composition of the membrane-bound fraction was at the level of 65–80% and changed non-monotonically. In the frequency range of 12–28 Hz, we recorded additional increase in oxyhemoglobin 10–30%. We assume that the level of oxyhemoglobin growth also depends on the initial amount of oxyhemoglobin, i.e. on the conditions of erythrocyte sedimentation from the blood. After increasing vibration amplitude to 0.90 ± 0.08 mm in the frequency range of 16–32 Hz, the content of oxyhemoglobin remained at a certain level for 120 minutes and decreased at the end of the experiment. In the experiments with 32 Hz frequencies, increase in the membrane-bound fraction of deoxyhemoglobin (up to 20%) in the membrane-bound fraction was recorded, which probably explains the increase in the content of membrane-bound hemoglobin in cells.

The content of methemoglobin (metHb) in erythrocytes increased significantly during exposure to the frequency range of 12–24 Hz, with the amplitude of 0.50 ± 0.04 mm and correlated with the activity of membrane-bound catalase. A direct correlation indicates the dependence of hemoglobin oxidation processes on O_2 level, rather than on the activity of antioxidant enzymes. The transition of methemoglobin to hemichrome was observed only during exposure to the frequencies of 28 and 32 Hz. In the conditions of exposure to the amplitude of 0.90 ± 0.08 mm, the content of methemoglobin in the cells was lower and decreased at the end of the experiment. Under these experimental conditions, we did not find a significant content of hemichrome in the membrane-bound fraction. Binding of metHb to the membrane is of physiological significance because it is reduced in the membrane region by membrane-bound NADPH-dependent metHb reductase (Kosmachevskaya et al., 2019).

Thus, we demonstrate that the targets of *in vitro* vibration are gas nanobubbles (babstons), which are on the cell surface and determine the content of available oxygen. Subject to the action of vibration, the nanobubbles first increase in size, causing increase in oxygen and oxidative load on the cells. Increasing the intensity or time of vibration leads to degassing of the fluid and the state of hypoxia. In this condition, oxidative processes in erythrocytes slow down (Mindukshev et al., 2019; Tang et al., 2019), while the processes aimed at restructuring the metabolism intensify. Therefore, in erythrocytes subjected to vibration with the amplitude of 0.90 ± 0.08 mm, the content of ATP and 2,3-diphosphoglycerate increases, which is characteristic of the state of hypoxia (Nemkov et al., 2018).

Can the described processes occur *in vivo*? We believe that probability exists, although, of course, this question needs to be explored in terms of a new paradigm. For many years, researchers have tried to look for resonant frequencies, the relationship between amplitude, frequency, duration of dosed vibration and the expressiveness of changes such as redox or other processes. The amount of information about clinical and experimental nature which describes the signs of vibration-induced symptoms at the level of various systems and organs increases annually. Nonetheless, this does not get us closer to understanding the mechanisms of influence of vibration on the human organism. We show that the effect of vibration is nonspecific in the range of 8–32 Hz. Depending on the intensity of its action, the level of oxygen in biological fluids changes. Prolonged vibration will lead to hypoxia. This conclusion coincides with the clinical symptoms of vibration. The pathogenesis of vibration is considered to be based on the hypoxia in the tissues (Dotsenko, 2014; Vorobeve & Shabanov, 2019; Vorobeve & Shabanov, 2020). It was previously demonstrated (Dotsenko, 2014) that the effect of vibration stimulated erythropoiesis in mice for two weeks, which is a compensatory phenomenon for the deve-

lopment of hypoxia, aimed at eliminating oxygen deficiency and excessive carbon dioxide from the body.

One should take into account that shaking and centrifugation are integral technical operations of laboratory tests. Prolonged centrifugation is always associated with vibration and, as a result, changes in the oxygen in the environment, which can affect the condition of the cells during their isolation or certain manipulations. The same applies to protein solutions that can change conformation and aggregate during shaking (Bunkin et al., 2020) on the principle of native aggregation.

Conclusion

The degree of binding of catalase and hemoglobin to the membrane of erythrocytes which were in buffer solution and subjected to vibration (frequency range 8–32 Hz, amplitudes 0.50 ± 0.04 and 0.90 ± 0.08 mm), changes in ligand forms in the content of the membrane-bound hemoglobin are dose-dependent.

The vibration affects nanobubbles of dissolved air (babstons), which are retained on the surface of erythrocytes due to Coulomb interactions and capable of coagulation and size increasing under vibration influence. These processes lead to increase in oxygen content on the surface of erythrocytes with consequent decrease as a result of degassing. Hemoglobin, as a sensor of oxygen content, can bind to certain centers on the membrane, thereby initiating the assembling of protein complexes that are able to respond to certain challenges. Increase in oxygen on the surface initiates redox reactions, and the decrease in oxygen – the restructuring of metabolic processes aimed at overcoming hypoxia.

Low-frequency vibration initiates O_2 -dependent processes in erythrocytes. The level of oxygen in the surrounding fluid is determined by the intensity and duration of vibration.

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