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PEROXIDE-INDUCED OXIDATIVE STRESS IN ERYTHROCYTES UNDER THE ACTION OF LOW-FREQUENCY VIBRATION

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Background. In recent years, many publications have highlighted the role of erythrocytes in the pathogenesis of various acute and chronic diseases. Their negative impact is explained by the ability of these cells to generate superoxide anion-radical and other reactive oxygen species (ROS) due to autooxidation of hemoglobin, which increases in hypoxia. The purpose of this work was to study the role of autooxidation of hemoglobin of erythrocytes and activation of redox processes in the regulation of specific physiological processes of these cells under the influence of vibration – a factor that combines mechanical influence, oxidative stress and hypoxia.

Materials and Methods. An erythrocyte suspension at T = 25 °C was subjected to vibration for 3 hours in the frequency range from 8 to 32 Hz, with amplitudes of 0.5±0.04 and 0.9±0.08 mm. At specified intervals of time, the content of hydrogen peroxide, the propensity of hemoglobin to autoxidation and the content of hemoglobin ligand forms of the cytoplasmic fraction in the hemolysates of cells were measured. Spearman's non-parametric correlation analysis was used to analyze the relationship between the studied indicators.

Results. The processes of hemoglobin autooxidation in erythrocytes under low-frequency vibration conditions were investigated. Changes in the kinetics of the reaction of erythrocyte hemoglobin oxidation with potassium hexacyanoferrate were observed. An increase in the content of intracellular hydrogen peroxide was shown, which increased more than twice in the frequency range of 16–24 Hz, A = 0.9±0.08 mm. The formation of hemichromes, an increase in the content of methemoglobin in cells was shown. In the frequency range of 20–32 Hz, the formation of ferrylhemoglobin was recorded.

Conclusion. Under the influence of vibration in the frequency range of 8–24 Hz, unstable forms of hemoglobin are formed in erythrocytes, which are oxidized to hemichromes. The process of hemoglobin autooxidation, which initiates oxidative stress, slows down over time due to the increase in the content of oxyhemoglobin. The formation of hemichromes at high frequencies indicates the involvement of hemoglobin in oxidative processes, which can have negative consequences for cells.

Keywords: ligand forms of hemoglobin, autoxidation of hemoglobin, heme, hypoxia, hemichrome, ferrylyhemoglobin, membrane-bound hemoglobin

INTRODUCTION

Erythrocytes are characterized by their remarkable deformability. Deformation stress occurs in the bloodstream even under physiological conditions (Nakamura, 2017; Liu & Nakamura, 2021). To cope with it, erythrocytes have well-established signaling mechanisms that convert mechanical stimulation into biochemical signals (Leo *et al.*, 2020; Liu & Nakamura, 2021; Ugurel *et al.*, 2022; Braidotti *et al.*, 2023; Cilek *et al.*, 2024) aimed at restoring cell's shape and volume.

It is known that under physiological conditions the erythrocyte membrane is in a state of chaotic oscillations (flickering) (Puckeridge *et al.*, 2014; Girasole *et al.*, 2023), which occur within a frequency range of 0.1–30 Hz. Research has shown that membrane flickering is caused by molecular bombardment, including that from external sources such as air bubbles. Additionally, metabolic processes also affect membrane flickering (Yoon *et al.*, 2009; Puckeridge *et al.*, 2014), so oscillations are a certain physical and mechanical response of the membrane to internal and external forces of various origins.

To investigate cellular responses to mechanical stress, we use stimulation of erythrocyte membrane oscillations under vibration conditions. Vibration, as a physical factor, is connected with the phenomena of cavitation and degassing (Ivanitsky *et al.*, 2023). It has been previously demonstrated that the concentration of air nanobubbles increases significantly following vibration treatment of water or solutions, with the size of the nanobubbles growing in accord with the vibration frequency and duration (Fang *et al.*, 2020). The average size of nanobubbles gradually increases with the duration of the vibration (Fang *et al.*, 2020; Ivanitsky *et al.*, 2023). As we have previously shown (Dotsenko *et al.*, 2021a; Dotsenko, 2023), at a certain frequency, amplitude and duration, vibration leads to a decrease in the content of dissolved O_2 and CO_2 through degassing, creating a state of hypoxia. Since erythrocytes function as oxygen sensors, their response to mechanical stress under these conditions is coupled with intracellular processes that aim to combat hypoxia.

In turn, the deformation of erythrocytes induces the release of vasoactive signaling molecules, such as nitric oxide (NO), ATP, superoxide anion radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) to regulate vascular tone (Rifkind *et al.*, 2018; Alayash, 2022; Cortese-Krott, 2023; Anastasiadi *et al.*, 2024). Unlike other types of cells, the main sources of $O_2^{-\cdot}$ and H_2O_2 in erythrocytes are the autooxidation reaction of oxyhemoglobin (HbO_2) to metHb and the processes of its further degradation (Kanias & Acker, 2010; Spolitac *et al.*, 2016; Rifkind *et al.*, 2018; Orrico *et al.*, 2018, Mahdi *et al.*, 2021; Orrico *et al.*, 2023).

Autooxidation of HbO_2 occurs spontaneously at a low rate, forming $O_2^{-\bullet}$, which is further transformed into hydrogen peroxide and oxygen. It is believed that under conditions of hypoxia, the hemoglobin autooxidation pathway acquires special importance (Mrakic-Sposta *et al.*, 2023; Orrico *et al.*, 2023; Yang *et al.*, 2024) and can cause negative consequences for the body (Mahdi *et al.*, 2021; Alayash, 2022; Yang *et al.*, 2024).

Accumulation of H_2O_2 can cause irreversible oxidative damage to hemoglobin molecules. H_2O_2 can react with deoxygenated hemoglobin (Hb), HbO_2 and methemoglobin metHb (metHb) to form metHb, ferrylhemoglobin (ferrylHb, [$HbFe^{IV}$ = O]) and oxoferrylhemoglobin (ferrylHb globin radical, ($Hb^{\bullet+}(Fe^{4+} = O^{2-})$), respectively (Kanias & Acker, 2010; Welbourn *et al.*, 2017; Nyakundi *et al.*, 2020; Potor *et al.*, 2021). The interaction of ferrylHb with peroxide impairs heme degradation due to the formation of superoxide in the hydrophobic heme pocket. This reaction forms a pathological cycle in which Hb cycles between the metHb and ferrylHb states, consuming hydrogen peroxide (Kanias & Acker, 2010).

Oxidized hemoglobin and, in particular, hemichromes formed by denatured hemoglobin have a much higher affinity for the erythrocyte membrane, forming irreversible cross-links, recruiting both band 3 and spectrin (Welbourn *et al.*, 2017; Barshtein *et al.*, 2024). Membrane-bound hemoglobin (MBH) affects membrane and whole-cell functionality, including cell surface charge, membrane permeability, and mechanical properties (Welbourn *et al.*, 2017; Barshtein *et al.*, 2024).

Binding of spectrin to hemoglobin and formation of H_2O_2-induced heme degradation products result in increased binding of IgG to the membrane (Welbourn *et al.*, 2017). When destroyed, these cells become a source of extracellular ferrylHb, which is internalized by macrophages. Macrophages exposed to ferrylHb were shown to exhibit a pro-inflammatory phenotype, as reflected in increased levels of IL-1β and TNF-α (Potor *et al.*, 2021).

The purpose of this work was to study the role of autooxidation of hemoglobin of erythrocytes and activation of redox reactions in the regulation of specific physiological functions of these cells under the conditions of vibration. We aim to determine how oxidative stress, as a fundamental adaptive reaction, becomes a pathological factor in hypoxia, forming several indirect effects on the biology of cells and the human body.

MATERIALS AND METHODS

The experimental protocol adhered to the principles of biological ethics and was approved by the Local Ethics Committee of the Faculty of Chemistry, Biology and Biotechnology at Vasyl' Stus Donetsk National University (Vinnytsia, Ukraine).

Erythrocytes obtained from the blood of healthy female donors of approximately the same age (45±5.5 years) were introduced into the environment of Na-phosphate buffer (0.01 M pH 7.4) containing 0.15 M NaCl. The resulting suspension of cells was subjected to low-frequency vibration for 3 hours in the frequency range from 8 to 32 Hz with a step of 4 Hz. The vibration amplitude (A) was maintained at the level of 0.5±0.04 and 0.9±0.08 mm. The hemoglobin content in the studied suspensions was 1.65±0.08 mg/mL. The conditions of the experiment are described in detail in the works (Dotsenko *et al.*, 2021a; Dotsenko *et al.*, 2021b; Dotsenko, 2023).

Every 15 to 20 min during the experiment, part of the suspension was taken, and the cells were precipitated. Hemolysate of erythrocytes was obtained by lysis of

cells in 0.01 M Na-phosphate buffer (pH 7.4) under cold conditions for 10 min. In the hemolysate, the content of intracellular H_2O_2 , ligand forms of cytoplasmic hemoglobin, and the propensity of hemoglobin to autooxidation were determined. Indicators obtained for erythrocytes that were not exposed to vibration and were in the same buffer solution were used as a control.

Determination of cytoplasmic H_2O_2 content. H_2O_2 content was determined after lysis of sedimented cells in 0.5 ml of cold water and subsequent precipitation of proteins with trichloroacetic acid (Ou & Wolff, 1994). The content was determined using the Fox reagent consisting of 100 μM Xylenol Orange, 250 μM ammonium ferrous sulfate, and 100 mM sorbitol in 25 mM H_2SO_4. The determination method is based on the ability to form a colored complex with the Fox reagent, which has an absorption maximum at 560 nm. The H_2O_2 content was determined using a calibration curve constructed for solutions of exact concentrations. The content in the cells was expressed in μM and related to the hemoglobin content in the sample, i.e., in μM/g Hb.

Study of the content of ligand forms of hemoglobin. The hemolysate was centrifuged (3000 rpm, 10 min) to separate the shadows. Absorption spectra of the cytoplasmic fraction of hemoglobin of erythrocytes were recorded in the wavelength range of 500–700 nm in cuvettes with a thickness of 1 cm.

The total content of hemoglobin in the cytoplasmic fraction was determined by absorption at a wavelength of 523 nm using an extinction coefficient of 7120 $M^{-1} \cdot cm^{-1}$ (Dotsenko *et al.*, 2021a). Absorption at 540, 560, 576, and 630 nm was used to study the content of oxy-, deoxy-, methemoglobin and hemichrome (Dotsenko *et al.*, 2021a). Ferrylhemoglobin (ferrylHb) content was calculated as described in Meng and Alayash (Meng & Alayash, 2017) with correction on hemoglobin tetrameric form. The content of each of the forms was expressed as a percentage in relation to the total content of hemoglobin in the cytoplasmic fraction.

Study of the kinetics of autooxidation of hemoglobin by ferricyanide. The reaction with potassium ferricyanide was used to determine the ability of hemoglobin to autooxidize (Rachmilewitz *et al.*, 1971). At low concentrations of ferric acid, the reaction rate constant is a measure of oxidation processes rather than displacement of oxygen from the protein.

Next, 0.2 mL of 30 μM potassium ferricyanide was added to 1.8 mL of 0.2 M Tris-HCl, pH 7.2. The reaction was initiated by adding 0.2 ml of erythrocyte hemolysate. The change in optical density at 540 nm was recorded for 4 min at 1-second intervals. The rate constant of the hemoglobin autooxidation reaction, which is a first-order reaction, was determined by the tangent of the slope angle of the dependence ln(*A*)-*t*, where *A* is the optical density of the solution, *t* is the time, s.

All experiments were performed in triplicate, and their results were analyzed using the Statistica 8.0 program (StatSoft Inc., USA). Experimental data are presented as x ± m (x is the mean, m is the standard error). Three-dimensional scatter plots were used to present the obtained experimental data. Based on the network of starting points, a surface was constructed using the method of inversely weighted distances. The statistical relationship between pairs of investigated indicators was evaluated using the Spearman rank correlation coefficient (r). Correlation coefficients were considered significant at the $p < 0.05$ level. The number of points was $n = 80$.

RESULTS AND DISCUSSION

Study of H_2O_2 content of erythrocytes under vibration conditions. Fig. 1*A* shows the change in H_2O_2 content compared to the control level depending on two factors: the frequency and time of vibration exposure with an amplitude of 0.5±0.04 mm. Vibration with frequencies of 8 and 12 Hz did not lead to a significant increase in H_2O_2 content compared to the initial level (with vibration at a frequency of 8 Hz, the initial H_2O_2 content in cells was 8.33±0.47 μM/g Hb, after 3 hours of exposure it was 10.18±0.23 μM/g Hb).

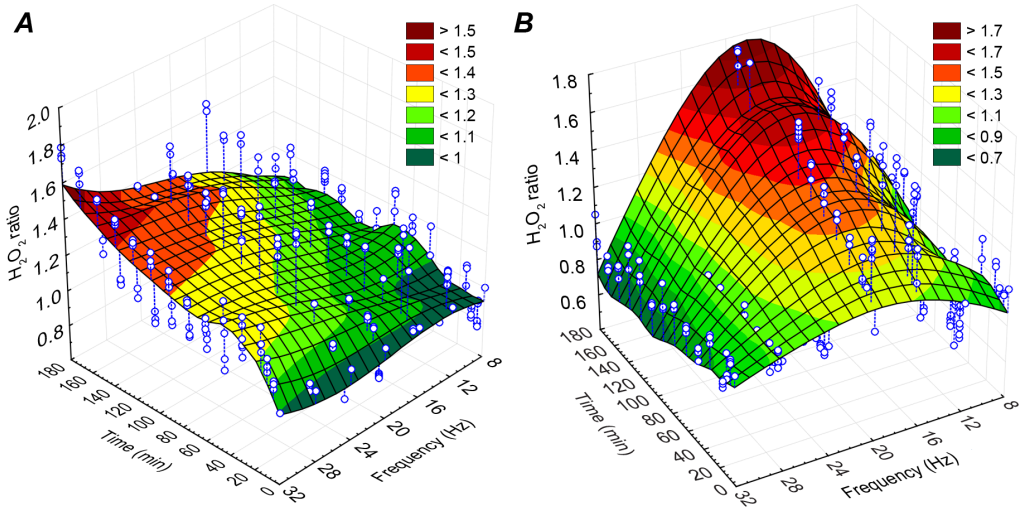

Fig. 1. The changes in the content of hydrogen peroxide in the cytosol of the cells exposed to vibration in the frequency range of 8–32 Hz with amplitudes of 0.50±0.04 mm (***A***) and 0.90 ± 0.08 (***B***) depending on the incubation time of cells. N (the number of points on the surface) = 180

Within the frequency range of 16–28 Hz, we observed a gradual accumulation of H_2O_2 content during vibration, which indicates the dose-dependent nature of this process and the absence of dependence on the frequency of vibration exposure (at the end of the experiment, the increase in H_2O_2 content was: 16 Hz – 1.61±0.27, 20 Hz – 1.34±0.01, 24 Hz – 1.36±0.02, 28 Hz – 1.22±0.06 times).

Fig. 1*B* shows the change in H_2O_2 content compared to the control when exposed to an amplitude of 0.9 ± 0.08 mm. Vibration in the frequency range of 16–24 Hz led to the maximum accumulation of H_2O_2 in erythrocytes (the increase was: 16 Hz – 2.27±0.07, 24 Hz – 2.1±0.09 times). In the frequency intervals of 8–12 Hz, the H_2O_2 content changed slightly. The relative increase in H_2O_2 content when exposed to a frequency of 8 Hz is 10–15 %, and when exposed to a frequency of 12 Hz did not exceed 20 %. In the frequency range of 28–32 Hz, there were no significant changes in H_2O_2 content compared to the initial level.

Study of the content of ligand forms of hemoglobin in the cytoplasmic fraction. During vibration with an amplitude of 0.5±0.04 mm in the frequency range of 8–24 Hz, a decrease in HbO_2 was observed during 90–100 min of exposure, the highest

values of which were observed during vibration with a frequency of 8 Hz (7.60±0.53 %) (**Fig. 2*A***). Under these conditions, the content of Hb increased to 3–5 %. As the vibration frequency increased, the percentage of HbO_2 content decreased (5.11±0.42 (12 Hz), 3.61±0.21 (16 Hz), 1.79±0.84 (20 Hz), 2.6±0.84 (24 Hz)). Further exposure led to an increase in the content of HbO_2 and a decrease in the content of Hb to the control level. Further exposure led to an increase in oxyhemoglobin to the control level. During vibration with frequencies of 32 and 28 Hz, the level of HbO_2 was at the control level or exceeded it by 1–2 %. The content of metHb in the cells changed slightly (**Fig. 3*A***) due to the activation of methemoglobin reductases (data not shown), while the content of hemichrome increased and at the end of the experiment was 0.3–0.5 %. Under vibration with frequencies of 28 and 32 Hz, the content of hemichrome, if it was present in the control, decreased to zero during the experiment.

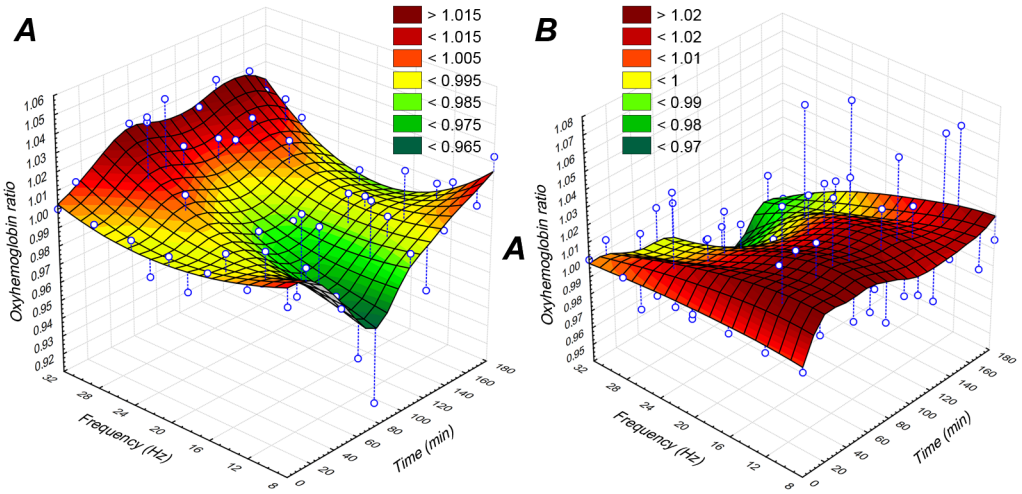

Fig. 2. The change of oxyhemoglobin content in the cytosol fraction of erythrocytes hemoglobin exposed to vibration in the frequency range of 8–32 Hz with amplitudes of 0.50±0.04 mm (***A***) and 0.90±0.08 (***B***) depending on the incubation time of cells. N (the number of points on the surface) = 80

FerrylHb was not present in the hemolysate of cells under the influence of vibration in the frequency range of 8–16 Hz. Starting from 20 Hz, the ferryl form begins to appear in the samples towards the end of the experiment, and in the range of 24–28 Hz, this form is present at all points. The highest content of ferrylHb (1.25±0.54 %) was recorded in experiments with a frequency of 28 Hz. Under conditions of vibration with a frequency of 32 Hz, FerrylHb was detected only at some time points during the experiment.

During vibration with an amplitude of 0.9±0.08 mm in the frequency range of 8–24 Hz, the content of HbO_2 in the cells increased during the 60–100 min of the experiment, then decreased to the control level (**Fig. 2*B***). The content of metHb (**Fig. 3*B***) and hemichrome increased. At the same time, the maximum amount of hemichrome did not exceed 0.5 %, and metHb – 15 % of the total amount of hemoglobin.

In the frequency range of 8-12 Hz, the increase in metHb at the end of the experiment was 18.78±2.55 %, hemichrome – 27.79±14.53 %. The greatest increase in metHb (44.92±8.13 %) was observed under the action of vibration with a frequency

of 24 Hz. The largest increase in hemichrome was observed under the action of vibration with a frequency of 16 Hz – 42.61±19.21 %. In the frequency range of 20–24 Hz, the level of hemichrome in the cells did not change significantly regarding the control level. Under the influence of vibration with frequencies of 28 and 32 Hz, a slight decrease in the content (5.08±1.07 %) of HbO_2 (**Fig. 2*B***) was observed 20–40 minutes after the start of the experiment, however the level of metHb in the cells did not change (**Fig. 3*B***). Hemichrome was detected only in some time points and its content was very low (≤ 0.1 %).

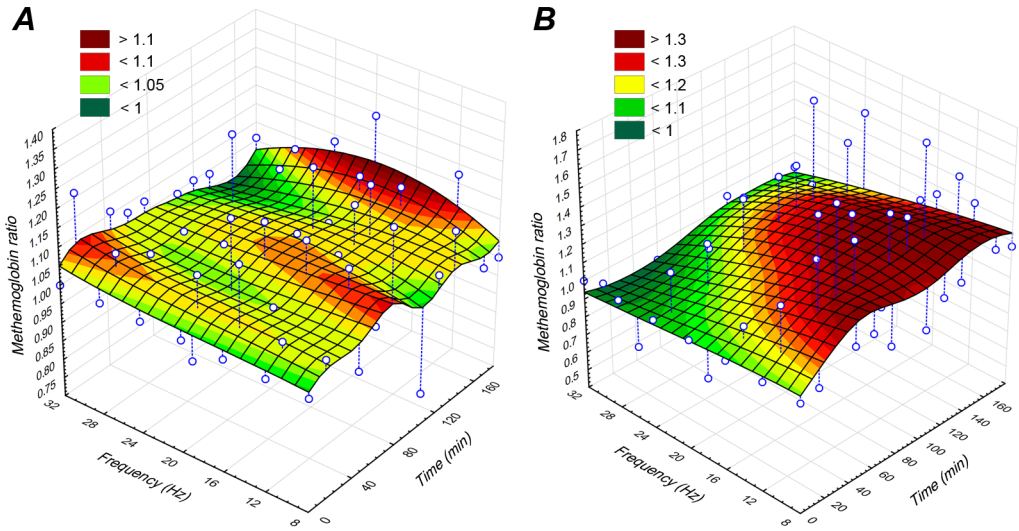

Fig. 3. The change of methemoglobin content in the cytosol fraction of erythrocytes hemoglobin exposed to vibration in the frequency range of 8–32 Hz with amplitudes of 0.50±0.04 mm (***A***) and 0.90 ± 0.08 (***B***) depending on the incubation time of cells. N (the number of points on the surface) = 80

The ferrylHb change followed a similar trend as in the experiments with an amplitude of 0.5±0.04 mm. However, under the action of vibration with a frequency of 28 Hz, the content of ferrylHb increased during 100 min by 2.24±0.95 times and further decreased almost to the control level. Under the influence of a 32 Hz frequency, ferrylHb was detected at some time points.

Study of the kinetic parameters of the hemoglobin oxidation reaction with potassium ferricyanide. The rate constant of hemoglobin oxidation varied from 7.5 to 15 s^{-1}, which indicates its strong dependence on the state of hemoglobin in the hemolysate of donor erythrocytes (Rachmilewitz *et al.*, 1971). **Fig. 4** shows the change in the rate constant k relative to the initial level.

Vibration in the frequency range of 8–24 Hz with an amplitude of 0.5±0.04 mm led to an increase in k at the beginning of the experiment. The time interval during which the value of k increased by 13–17 % from the initial level was 60–105 min and decreased with increasing exposure frequency. Further influence of the factor led to a decrease in k, which was: 5.80±3.95 % (8 Hz), 7.97±6.08 % (12, 16 Hz), 13.0±5.51 % (20 Hz), 25.65±9.64 % (24 Hz), 15.97±10.78 % (28 Hz). When subjected to vibration

with a frequency of 32 Hz, a monotonous decrease in k was observed from the beginning of the experiment. At this amplitude, it was 29.64±21.09 %.

Under vibration with an amplitude of 0.9±0.08 mm, a decrease in the rate constant was observed from the beginning of the experiment. The decrease in the rate constant relative to the control level at the end of the experiment was: 9.78±7.72 % (8 Hz), 29.21±12.16 % (12 Hz), 24.59±11.79 % (16 Hz), 37.54±16.12 % (20 Hz), 28.75±17.19 (24 Hz), 32.92±18.66 % (28 Hz), 34.58±23.15 % (32 Hz). The change in autooxidation k under the action of vibration with an amplitude of 0.9±0.08 mm has a stepwise character (**Fig. 4*B***), which indicates additional factors that slow down the autooxidation of hemoglobin by ferricyanide.

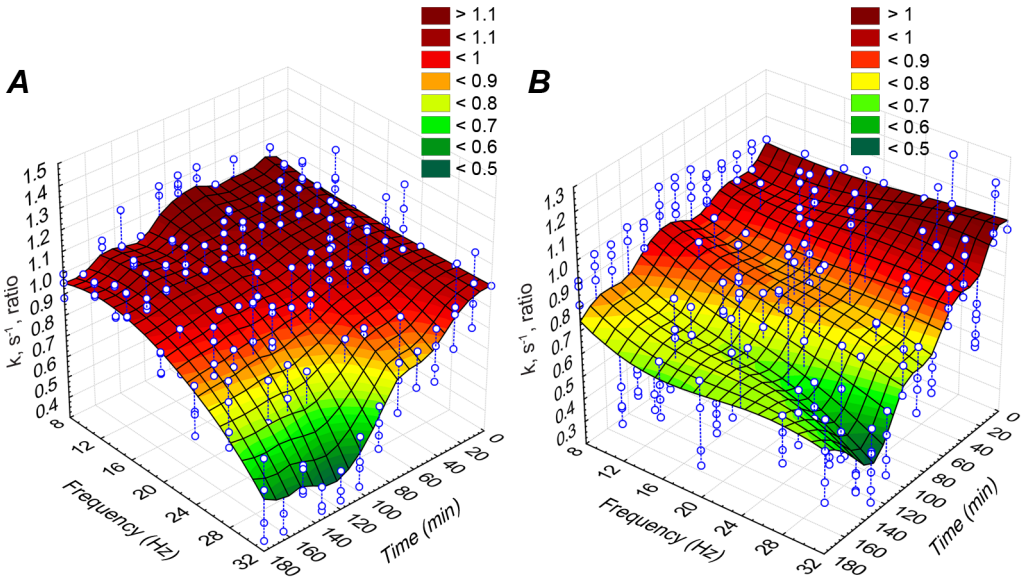

Fig. 4. The changes in the rate constant (k, s^{-1}) of hemoglobin oxidation by potassium ferricyanide exposed to vibration in the frequency range of 8–32 Hz with amplitudes of 0.50±0.04 mm (***A***) and 0.90±0.08 (***B***) depending on the incubation time of cells. N (the number of points on the surface) = 180

Correlation between system elements. To understand the relationships between the obtained experimental data, we conducted a correlation analysis between H_2O_2 content and hemoglobin ligand forms. In the analysis, we included data on the formation of membrane-bound hemoglobin (MBH), which were described earlier (Dotsenko *et al.*, 2021a).

The correlations between the above indicators for vibration in the range of 8–24 Hz, with an amplitude of 0.5±0.04 mm are similar, therefore, as an example, we present the correlation coefficients for the data obtained in the experiment with a frequency of 16 Hz (**Fig. 5*A***). We found significant negative relationships between HbO_2 content and Hb (r = -0.65), HbO_2 and metHb (r = -0.68) (**Fig. 5**). Thus, in the frequency range of 8–16 Hz, the decrease in the content of HbO_2 occurred due to its oxidation and transition to metHb and hemichrome (**Figs. 2*A***, **3*B***). H_2O_2 content was inversely correlated with HbO_2 (r = -0.75) and directly with metHb (r = -0.68).

We found a significant negative correlation between MBH content and HbO_2 (r = -0.81), and a positive correlation between MBH content and metHb and H_2O_2 (r = 0.64, r = 0.65, respectively).

Under vibration in the frequency range of 28–32 Hz with an amplitude of 0.5±0.04 mm, the connections between HbO_2, metHb and H_2O_2 disappear, although the content of H_2O_2 in erythrocytes increases. The correlations between the ligand forms of hemoglobin are lost, the correlation coefficient between MBH and HbO_2 changes sign (r = 0.72).

Connections between FerrylHb, which was detected in the cytosol of cells under the influence of vibration with frequencies of 20 and 24 Hz with other components of the system were not found. Under exposure to vibration with a frequency of 28 Hz, correlations were found between FerrylHb and HbO_2 (r = -0.70) and FerrylHb and metHb (r = 0.91).

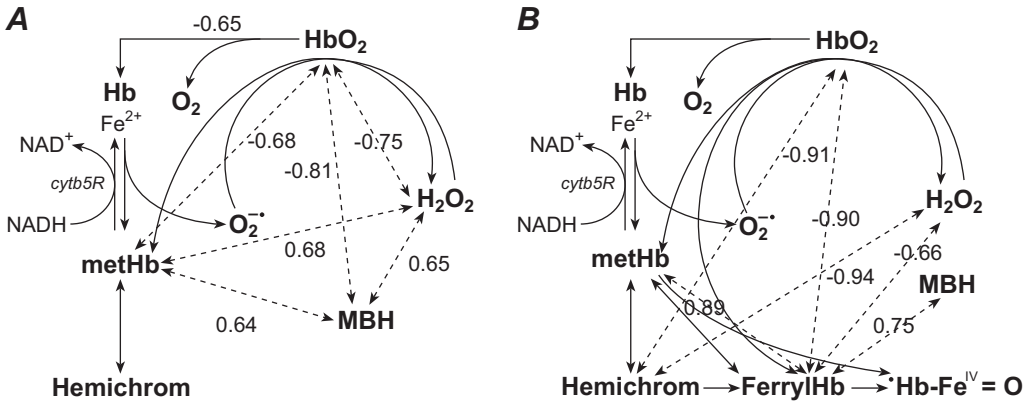

Fig. 5. Correlations between the erythrocytes content of H_2O_2, ligand forms of hemoglobin and membrane-bound hemoglobin (MBH) under the influence of vibration with a frequency of 16 Hz, an amplitude of 0.50±0.04 mm (***A***) and vibration with a frequency of 28 Hz, an amplitude of 0.90±0.08 mm (***B***)

When the amplitude increases (0.9±0.08 mm) in the frequency range of 8–20 Hz, the correlation between metHb and H_2O_2 disappears, while the connections between Hb, metHb and hemichrome are preserved. Starting from 20 Hz, correlations appear between ferrylHb and metHb and HbO_2 (r = 0.82 and r = 0.80, respectively), and a weak connection with H_2O_2 (r = -0.60). The inverse correlation between ferrylHb and H_2O_2 was shown in (Nyakundi *et al.*, 2020). As the frequency of exposure increases, these connections become stronger. For example, **Fig. 5*B*** presents correlations between system components under conditions of 28 Hz.

Discussion. Autooxidation of hemoglobin takes place with the participation of hemoglobin-bound molecular oxygen and is directed exclusively at the Fe^{2+} ion of the prosthetic group of heme, which leads to the formation of superoxide radical and methemoglobin. This reaction proceeds at a low speed, therefore oxidants are used to study the propensity of hemoglobin to autoxidation.

The resistance of HbO_2 to the action of chemical oxidants expresses the reactivity of heme, which may be related to one or another conformation of the protein molecule, the availability of the heme pocket to the solvent. It is known that the distal *His*

plays a role in regulating the accessibility of the heme pocket to external oxidants (Tsuruga *et al.*, 1998). Acceleration of the oxidation process of HbO_2 by ferricyanide, observed at the beginning of low-amplitude experiment, can be associated with a decrease in cytoplasmic pH and protonation of the distal protein of the *His* molecule, which enables the entry of water into the heme pocket and contributes to the autooxidation of hemoglobin.

The specific quaternary structure is necessary not only for efficient oxygen binding, but also for its molecular stability (Rachmilewitz *et al.*, 1971; Tsuruga *et al.*, 1998; Shikama & Matsuoka, 2003). It is known that there are considerable differences in the rate of autooxidation of a and b chains, therefore, hemoglobin autooxidation occurs with the formation of two intermediate forms, which are valence hybrids of hemoglobin $(\alpha^{3+}\beta^{2+})_2$ and $(\alpha^{2+}\beta^{3+})_2$. Abnormal hemoglobin molecules with unusual oxygen-binding properties are themselves more prone to oxidation than oxyhemoglobin A (Tomoda *et al.*, 1981). Note, that hemichromes originate from unstable hemoglobin, which has a greater tendency to spontaneously oxidize to methemoglobin and hemichrome. The formation of these forms can also account for the growth of k autooxidation.

The transformation of HbO_2 into hemichrome is always accompanied by the formation of the high-spin form of ferrihemoglobin. The stage of formation of the high-spin form of ferrihemoglobin is rate-limiting. For oxyhemoglobin A, this stage occurs very slowly (Rachmilewitz *et al.*, 1971). We observed that a decrease in the rate constant (k) of the autooxidation reaction correlates with an increase in the content of oxyhemoglobin in the cytosol.

If the mechanism of autoxidation of HbO_2 can be represented by the following sequence of reactions (Mal & Chatterjee, 1991; Kanias & Acker, 2010):

$$HbO_2 \rightleftharpoons Hb^{3+}O_2^{-\cdot},$$

$$Hb^{3+}O_2^{-\cdot} \longrightarrow metHb + O_2^{-\cdot},$$

$$HbO_2 + O_2^{-\cdot} + 2H^+ \longrightarrow metHb + H_2O_2 + O_2,$$

$$HbO_2 + H_2O_2 \longrightarrow metHb + {}^{\cdot}OH + OH^- + O_2,$$

$$HbO_2 + {}^{\cdot}OH \longrightarrow metHb + OH^- + O_2,$$

then the total reaction can be represented as:

$$4HbO_2 + 2H^+ \longrightarrow 4metHb + 2OH^- + 3O_2.$$

That is, 3 mol of O_2 is released for 1 mol of heme that autoxidizes to the met form. We assume that autooxidation is limited by a certain protective mechanism that ensures oxygenation of cytoplasmic hemoglobin in the absence of oxygen. $O_2^{-\cdot}$, formed as a result of dissociation, $Hb^{3+}O_2^{-\cdot}$ is not completely inactivated by enzymes. According to A. Mal and I. B. Chatterjee (1991), autooxidation of hemoglobin is inhibited by SOD by only 31 % and 53 % by catalase.

Therefore, there is a high probability of HbO_2 interaction with the formation of metHb and H_2O_2. H_2O_2 can oxidize HbO_2 again. Thus, intracellular H_2O_2 is a product of autooxidation of hemoglobin and can in a certain way reflect the intensity of this process (**Fig. 1**) under conditions of absence (or slight activation) of pseudoperoxidase activity of hemoglobins oxidized forms. According to our data (**Fig. 4**), the formation of endogenous oxygen contributes to the normalization of HbO_2 content, overcoming hypoxia and slowing down autooxidation.

It was shown (Potuznik *et al.*, 1993; Tsuruga *et al.*, 1998) that the previous modification of the sulfhydryl groups of Cys-93β can reduce the efficiency of oxidation of HbO_2 by ferricyanide, inhibiting the transfer of an electron from the Fe^{2+} heme to the ferricyanide molecule. The electron transfer from the radical from the heme pocket to the outside, to the surface thiol, probably occurs through the electron delocalized system of the proximal *His*, which is in the same part of the α-helix as Cys-93β, only on its opposite outer side (Potuznik *et al.*, 1993). The modification of Cys-93β SH-groups can accelerate the process of autoxidation of HbO_2, increasing its affinity to oxygen and inhibiting its oxidation by ferricyanide. The modification of SH-groups may interfere with the interaction of ferricyanide with Cys-93, which is the primary stage of ferricyanide binding by hemoprotein.

Thus, an increase in the amplitude and frequency of vibration leads to oxidative modification of hemoglobin. In this regard, the reaction with ferricyanide can be useful for the analysis of structural changes in the hemoglobin molecule, but it does not allow judging the intensity of autoxidation.

It is known that a decrease in exogenous oxygen increases the autoxidation of hemoglobin (Rifkind *et al.*, 1991; Rifkind *et al.*, 2004). We have previously shown (Dotsenko *et al.*, 2021a; Dotsenko, 2023) that the vibration effect in the frequency range of 28–32 Hz leads to a decrease in oxygen in the incubation medium and the development of hypoxia in the cells, which intensifies with an increase in the vibration amplitude.

According to the obtained results, starting from 20 Hz, ferrylHb is detected in cell hemolysates. It is known that superoxide ions and hydrogen peroxide formed during the autoxidation of HbO_2 induce two-electron oxidation of the latter to form ferrylHb, in which the formal charge of heme iron is +4 (Kanias & Acker, 2010; Meng & Alayash, 2017; Nyakundi *et al.*, 2020). FerrylHb is an unstable form that quickly turns into metHb, therefore it is difficult to detect by spectrophotometric methods (Nyakundi *et al.*, 2020; Potor *et al.*, 2021). FerrylHb can react with an additional molecule of H_2O_2, which is reduced to form a superoxide radical that remains in the heme pocket long enough to react with heme, ultimately leading to heme degradation (Kanias & Acker, 2010; Rifkind & Nagababu, 2013).

In addition, the reaction of metHb with H_2O_2 leads to the formation of reactive ferrylHb globin radical ($Hb^{\bullet+}(Fe^{4+} = O^{2-})$), in which the formal charge of heme iron is +4, and the unpaired electron is located on the globin chain (Kanias & Acker, 2010). Protein radicals migrate, causing further damage to the protein, including the oxidation of bCys-93 and dimerization. FerrylHb tends to form free radicals in the a (αTyr-24, aHis-20, αTyr-42) and b (βTyr-36, βTyr-130) chains of globins (Potor *et al.*, 2021). Termination reactions of these radicals result in the formation of globin-globin crosslinks, leading to hemoglobin dimers, tetramers, and multimers. (Potor *et al.*, 2021; Nyakundi *et al.*, 2020).

According to the obtained data, the content of H_2O_2 in erythrocytes significantly increases under the conditions of vibration with an amplitude of 0.9±0.08 mm, however, during vibration with frequencies of 28 and 32 Hz, the content of H_2O_2 is much lower (**Fig. 1*B***). Earlier, we showed that under these conditions the peroxidase activity of hemoglobin significantly increases (Dotsenko *et al.*, 2021b), especially at frequencies of 28 and 32 Hz. Based on the obtained data, it is clear that under these conditions (28, 32 Hz, A = 0.9±0.08 mm) this process represents a pseudoperoxidase cycle and takes

place with the participation of metHb and ferrylHb (Spolitak *et al.*, 2016). The pseudoperoxidase activity of metHb and ferrylHb may cause this decrease.

The conducted correlation analysis (**Fig. 5**) shows that under the experimental conditions of 8–16 Hz, the accumulation of MBH is stimulated by relatively low-intensity oxidative processes. We recorded an increase in methemoglobin content in MBH (Dotsenko *et al.*, 2021a). Under exposure to vibration of 28, 32 Hz, A = 0.9±0.08 mm, the content of MBH is statistically significantly correlated only with FerrylHb. It is known that unstable, oxidized and radical forms of hemoglobin have an increased affinity for the membrane and bind to it irreversibly (Welbourn *et al.*, 2017). As shown (Welbourn *et al.*, 2017), hemoglobin initially remains in a redox active functional form, reverting over time to irreversible dysfunctional hemichrome complexes. We previously recorded a large amount of hemichrome in MBH of erythrocytes subjected to vibration under these conditions (Dotsenko *et al.*, 2021a).

Partial oxygenation of hemoglobin increases its affinity to the membrane and helps to increase the rate of autoxidation of hemoglobin in the membrane (Rifkind & Nagababu, 2013). During autoxidation MBH, $O_2^{-\cdot}$ is formed, which is believed to be transported outward, bypassing the antioxidant defense system. Mechanisms of $O_2^{-\cdot}$ transport formed during autooxidation of MBH are discussed in works (Rifkind *et al.*, 2018; Mahdi *et al.*, 2021), but not confirmed experimentally. However, it is a proven fact that the binding of antioxidant defense enzymes to the membrane is enhanced during oxidative stress and enzymopathies (Dotsenko *et al.*, 2020; Melo *et al.*, 2023).

We assume that the toxicity of $O_2^{-\cdot}$, produced by erythrocytes during hypoxia is exaggerated. More negative consequences may include the accumulation of nonfunctional hemoglobin on the surface of cells, which makes them the target of innate immune responses by phagocytes and natural antibodies (Beppu *et al.*, 1990; Anderson *et al.*, 2018). Then all the clinical and biochemical signs of inflammation described in the literature (Kiefmann *et al.*, 2008; Gwozdzinski *et al.*, 2021; Mahdi *et al.*, 2021; Potor *et al.*, 2021; Obeagu *et al.*, 2024) can be observed.

CONCLUSION

The intensity of oxidative processes involving hydrogen peroxide and hemoglobin depends on the frequency and amplitude of the vibration.

It was observed that during a 3-hour exposure to vibrations in the frequency range of 8–24 Hz the reduced content of HbO_2 was either restored to control levels or increased from the beginning of the experiment (A = 0.9±0.08 mm). However, hemichrome was present in the cell hemolysates, and ferrylhemoglobin was detected starting at 20 Hz. With vibrations at A = 0.9±0.08 mm, the level of methemoglobin in cell hemolysates increased to 44.92±8.13 %.

It was established that the content of H_2O_2 significantly increased in cells exposed to vibrations in the frequency range of 16–32 Hz, with A = 0.5±0.04 mm. The maximum accumulation of H_2O_2 (more than 2 times) was observed under conditions of 16–24 Hz, A = 0.9±0.08 mm.

Under vibrations at frequencies of 28 and 32 Hz, with A = 0.9±0.08 mm, a slight decrease in the content of oxyhemoglobin (5.08±1.07 %) was observed. The H_2O_2 content did not change under these conditions. The experimental data suggest that [specific substance] is involved in pseudoperoxidase cycles with oxidized forms of hemoglobin.

Additionally, the vibration effect in the studied frequency range reduces the rate of auto-oxidation of hemoglobin by ferricyanide, indicating oxidation of sulfhydryl groups in the hemoglobin molecule.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Human Rights: all studies were conducted following the guidelines outlined in the Declaration of Helsinki. Approval for the study was obtained from the Ethics committee of Vasyl' Stus Donetsk National University (protocol No. 2 of January 11, 2021).

AUTHOR CONTRIBUTIONS

Conceptualization, [O.D.; G.T.]; methodology, [O.D.]; validation, [O.D.; G.T.]; formal analysis, [G.T.]; investigation, [O.D.; G.T.]; resources, [O.D.; G.T.]; data curation, [O.D.; G.T.]; writing – original draft preparation, [O.D.]; writing – review and editing, [O.D.; O.D.]; visualization, [D.K.]; supervision, [O.D.; G.T.].

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ПЕРОКСИД-ІНДУКОВАНИЙ ОКИСНИЙ СТРЕС В ЕРИТРОЦИТАХ В УМОВАХ НИЗЬКОЧАСТОТНОЇ ВІБРАЦІЇ

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Вступ. Останніми роками з'явилась велика кількість публікацій про участь еритроцитів у патогенезі різноманітних гострих і хронічних захворювань. Їхня негативна роль пояснюється властивістю цих клітин генерувати супероксид аніон-радикал та інші АФО під час аутоокиснення гемоглобіну, яке посилюється у стані гіпоксії. Мета роботи полягала в дослідженні ролі аутоокиснення гемоглобіну еритроцитів і активації окисно-відновних процесів у регуляції специфічних фізіологічних процесів цих клітин в умовах дії вібрації, яка є чинником, що поєднує механічний вплив з окисним станом та гіпоксією.

Матеріали та методи. Суспензію еритроцитів піддавали дії вібрації протягом 3-х годин в інтервалі частот від 8 до 32 Гц амплітудами 0,5±0,04 та 0,9±0,08 мм. Через певні проміжки часу в гемолізатах клітин визначали вміст гідроген пероксиду, схильність гемоглобіну до аутоокиснення та вміст лігандних форм цитоплазматичної фракції гемоглобіну. Непараметричний кореляційний аналіз Спірмена застосований для аналізу взаємозв'язків між дослідженими показниками.

Результати. Досліджено процеси аутоокиснення гемоглобіну еритроцитів в умовах низькочастотної вібрації. Виявлено зміни у кінетиці окиснення гемоглобіну еритроцитів калій гексаціанофератом. Встановлено зростання вмісту внутрішньоклітинного гідроген пероксиду, яке у діапазоні частот 16–24 Гц, A = 0,9±0,08 мм збільшувалось більш ніж удвічі. Встановлено утворення геміхромів, зростання вмісту метгемоглобіну у клітинах. В інтервалі частот 20–32 Гц фіксується утворення ферилгемоглобіну.

Висновки. За дії вібрації в інтервалі частот 824 Гц в еритроцитах утворюються нестабільні форми гемоглобіну, які окислюються до геміхромів. Процес аутоокиснення гемоглобіну, що ініціює окисний стрес, уповільнюється з часом

експерименту через підвищення вмісту оксигемоглобіну. Утворення геміхромів в інтервалі високих частот свідчить про залученість гемоглобіну до окисних процесів, що може мати негативні наслідки для клітин.

Ключові слова: лігандні форми гемоглобіну, аутоокиснення гемоглобіну, гем, гіпоксія, геміхром, ферилгемоглобін, мембранозв'язаний гемоглобін

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