Alternating Magnetic Field Effect on Oxygen Transport Function of Red Blood Cells *in Vitro*

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Abstract. The study presents data on the influence of alternating magnetic field (AMF) on blood *in vitro*, manifested by a decrease in hemoglobin's affinity for oxygen and an increase in nitrate/nitrite content. The effect of short-term exposure (30 min) to AMF with the strength of 300 mT and frequency of 20 Hz (corresponding to the radiation from static magnetic field sources) on the oxygen transport function (OTF) of erythrocytes and conformation of Fe-containing hemoporphyrin and globin of hemoglobin was demonstrated using Raman spectroscopy. Alteration in the content of hemoglobin in the T and R-forms was revealed, indicating a different affinity of hemoglobin for ligands and associated with changes in the polarity of the globin amino acids. It was also demonstrated that exposure of erythrocytes to AMF for 360 s modifies their gasotransmitter-forming function. These changes can significantly influence the alteration of blood OTF and change the blood flow in meeting tissue oxygen demands. The obtained results indicate the point to the involvement of the gasotransmitter-forming function in modifying blood OTF and, consequently, in altering the adequacy of blood flow in meeting tissue oxygen demands.

Keywords: oxygen transport function; magnetic field; erythrocyte; Raman spectroscopy.

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1 Introduction

The adaptive capabilities of erythrocytes are limited by cytoplasmic mechanisms, in which hemoglobin (Hb) plays a key role. It serves as a temporary transport depot for oxygen and possesses a number of properties that ensure both the intensive formation of oxygenated hemoglobin and the timely release of O_2. The state of the cytoplasm system of regulating Hb's affinity for oxygen significantly influences the mobility of changes in blood. At the same time, this autonomous system, operating on a feedback principle, is capable of maintaining the adaptive properties of blood independently of circulatory conditions. In the real conditions of the organism during hypoxia, the action of various modulators causes changes in Hb's affinity for oxygen. This is particularly important for ensuring adequate oxygen flow to tissues and can be used to eliminate oxygen deficiency [1].

Impaired tissue oxygenation underlined the pathogenesis of a wide range of diseases, from cardiovascular and chronic lung diseases to critical conditions accompanied by multiple organ dysfunction syndrome. One of the factors reducing treatment effectiveness is tissue hypoxia. It is known that the oxygen transport function of blood is determined by the interaction of erythrocytes with other cells, plasma components, and vascular cells [2]. The main component of red blood cells that transports oxygen is the tetrameric protein hemoglobin (Hb), which contains Fe^{2+}-protoporphyrin IX (heme). Heme is known to exist in two conformational states [3, 4]: when Hb binds oxygen, a conformational change occurs, increasing Hb's affinity for molecular oxygen. This model of cooperative O_2 binding is based on two Hb states:

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– R-state ("flat" heme conformation) with high affinity for oxygen-oxyhemoglobin (oHb);

– T-state ("domed" (non-planar) conformation) with low affinity for oxygen-deoxyhemoglobin (dHb).

Depending on the number of O_2 molecules bound by Hb subunits, local concentrations of H^+ ions, CO_2, etc., it is possible to observe the R-state of heme not bound to O_2 and the T-state conformation of heme bound to O_2. The transition between T and R states is a trigger that initiates a series of structural cooperative changes of globin during the dissociation (or binding) of O_2 [5]. In this case, oHb is characterized by higher packing density globule structure than dHb [6]. For non-invasive assessment of the oxygen transport function (OTF) and conformation of Hb molecules in the cytoplasm of erythrocytes, Raman spectroscopy is widely used [6–9].

One of the promising methods for influencing the erythrocytes OTF is low-intensity magnetic field (MF) exposure, which demonstrates a pronounced anti-hypoxic effect: improved tissue oxygenation and reduced Hb affinity for oxygen (facilitating O_2 release in the microcirculatory bed). The sensitivity of erythrocytes to a magnetic field (100–600 mT) has been previously demonstrated: the binding of oxygen to Hb affects its magnetic susceptibility, reduces blood viscosity, leads to an increase in osmotic fragility, a decrease in the elasticity of the plasma membrane and a change in blood flow velocity [10–13].

Furthermore, prolonged or high-intensity magnetic field exposure can affect the tertiary structure of the protein, which in turn influences Hb's affinity for ligands [14]. However, the operation of molecular-cellular mechanisms following magnetic field exposure is still not fully understood. Studies [15, 16] discuss the hypothesis of mediated regulation through the gasotransmitter systems of erythrocytes (nitric oxide NO and hydrogen sulfide H_2S). These systems can alter the hemoglobin conformation through the formation of nitroso- and sulfo-derivatives, thereby changing its affinity for oxygen.

The goal of this work is aimed at studying the influence of short-term exposure of erythrocytes to alternating magnetic field (AMF) (30 min, 300 mT, 20 Hz) on cytoplasmic Hb. As a control for changes in heme conformation, purified Hb isolated from erythrocytes (isolated Hb) was used.

2 Materials and Methods

2.1. Experiments with Rat Blood

2.1.1 Isolation Erythrocytes from Rat Blood

Blood samples were collected on white male-rats weighing 250–300 g. Animal manipulations were performed under conditions of adequate analgesia in accordance with the recommendations and decision of the Committee on Biomedical Ethics of Grodno State Medical University. For 7–10 days prior to each stage of the study, the animals were acclimatized to vivarium conditions. The studies were conducted in the first half of the day.

Erythrocytes were obtained by separating the whole blood samples into plasma and leukocyte- and platelet-depleted mass of erythrocytes (at 1500 g), followed by triple washing of the erythrocytes with the isotonic 0.9% NaCl solution.

2.1.2 Effect of Alternating Magnetic Field (AMF)

The "UniSPOK" with the IAMV-4 inductor (LLC "MagnoMed", Belarus) was used as the AMF source. With the pulsed current with a frequency 60–200 Hz, modulated at 10 Hz, the magnetic induction was 150 mT.

In the first series of experiments, AMF exposure was applied to the erythrocyte suspensions (in isotonic 0.9% NaCl solution or blood plasma) for 360 s. In the second series of experiments, 1 ml of whole blood was exposed to AMF with different durations (120 and 360 s). As a control, the erythrocytes with added isotonic 0.9% NaCl solution or plasma depleted of leukocytes and platelets were used. The number of replicates was 9.

2.1.3 Oxygen Transport Function (OTF) of Blood

OTF indicators were determined at 37 °C using the micro-gas analyzer "Synthesis-15" (Instrumentation Laboratory, USA) based on the measurement of p50 parameter (pO_2 at 50% blood oxygen saturation) by the spectrophotometric method at actual values of temperature, pH, and pCO_2 ($p50_{real}$). Parameter $p50_{stand}$ was calculated using the J. W. Severinghaus formulas [17]. Based on the $p50_{real}$ and Hill equation, the position of the oxyhemoglobin dissociation curve was calculated. NO production was assessed by the concentration of nitrate/nitrites (NO_3^-/N O_2^-) using the Griess reagent [18] on a PV1251C spectrophotometer (Solar, Belarus) at the wavelength of 540 nm.

2.2 Experiments with Human Blood

2.2.1 Isolation Erythrocytes from Human Blood

Blood from healthy donors was collected from the cubital vein into vacuum tubes (Vacuette, Russia) with heparin (20–50 U/mL blood) as an anticoagulant and stored at 4°C for 3 h after collection. The erythrocyte fraction was sedimented by centrifuging the blood at 1500 g for 5 min at 4°C (Laborfuge 400R, Thermo Scientific, USA). The resulting supernatant was removed, and the pellet was washed in Allen's solution (145 mM NaCl, 5 mM KCl, 4 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 5 mM glucose, 1 mM $CaCl_2$ (Sigma, USA), pH 7.4). The washing procedure was repeated three times. Washed erythrocytes were suspended in Allen's solution (Ht = 40%), stored at 4 °C and used in the experiment within 3 h after isolation.

Experiments with human blood were conducted in accordance with the principles of biomedical ethics as formulated in the Declaration of Helsinki of 1964 and its subsequent amendments and were approved by the local bioethics committee of the Lomonosov Moscow State University.

2.2.2 Isolation of Hb from the Erythrocytes

Phosphate buffer (pH 7.4) was added to the erythrocytes in a 1:10 ratio. The suspension was thoroughly mixed and centrifuged at 6000 g for 10 min at +4 °C. The supernatant was separated and stored at +4 °C. The sample was used within 3 h after isolation.

2.2.3 Exposure to an Alternating Magnetic Field (AMF)

A magnetic device (Department of Biophysics, Lomonosov Moscow State University, Russia) was used to generate the AMF: magnetic induction 300 mT, rotation frequency ~20 Hz). When studying the effect of AMF on Hb, the magnetic device was positioned above the capillary at a distance of ≈ 1.5 cm. The exposure time was 30 min. Changes in the conformation of the heme and globin were recorded after 1 min. During Raman signal acquisition, the magnetic device was turned off. All experiments were performed at room temperature.

2.2.4 Raman Spectroscopy (RS)

The signal from cytoplasmic Hb was recorded by the NTEGRA-SPECTRA confocal microscope-spectrometer (NT-MDT, Russia) in the range of 1000–3000 cm^{-1}, grid: 600 lines per mm with a measurement step of 0.8 cm^{-1}. CCD detector with Peltier cooling to –50°C was used (objective 5× with an aperture of 0.15 (Olympus, Japan)). Line excitation was 532 nm (2 mW per 10–12 μm laser spot size to record resonance Raman scattering of Hb in individual erythrocytes), the recording time for one spectrum was 10 s. Measurements were performed at room temperature (22–25 °C). To record the Raman spectra, the sample (Hb or erythrocytes) was placed in a hematocrit capillary (Agat-Med, Russia) with a cross-sectional diameter of 1 mm. The spectrum processing included baseline subtraction and smoothing with Origin2021 (Origin-Lab Corp., United States).

To analyze conformational changes of the heme and globin in Hb the following ratios of characteristic Raman bands were used [6, 19–21]:

I_{1375}/I_{1127} – contribution of lateral $–CH_3$ groups of pyrrole half-rings in hemoporphyrin. It changes along with the changes in globin conformations in the immediate vicinity of the heme. It characterizes the manifestation of symmetrical and asymmetrical vibrations of pyrrole half-rings (a decrease indicates an increase in heme mobility and the proportion of oxyhemoglobin);

I_{1375}/I_{1172} – contribution of the asymmetrical and symmetrical vibrations of pyrrole rings in hemoporphyrin (depends on the action of the protein environment of globin and on changes in heme pyrroles);

I_{1580}/I_{1550} – contribution of the vibrations of methine bridges between pyrroles in hemoporphyrin emerging in macrocycle deformations (an increase indicates a rise of the Hb affinity to ligands);

$I_{1375}/I_{(1355+1375)}$ – increase indicates an increase in the relative amount of oxygenated Hb complexes;

I_{1618}/I_{1580} – contribution of the stretching vibrations (C_1C_2) of vinyl groups relative to C_aC_mH, methine bridge vibrations. An increase indicates an increase in the relative amount of Hb complexes with NO (the Hb–NO(I) complex);

I_{2850}/I_{2880} – contribution of the symmetrical vibrations of methylene groups in –CH amino acids of globin (an increase characterizes a rise of the packing density of amino acids);

I_{2880}/I_{2930} – contribution of asymmetric vibrations of C-H stretch vibrations from free amino acid side-chain CH_2 groups of amino acids (an increase characterizes a rise in the orderliness and packing density of amino acids).

Due to the fact that rat blood has less stable parameters during long-term measurements, the work was carried out on human blood using the Raman method [22].

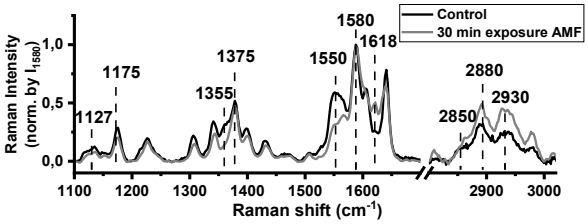

Fig. 1 Raman spectrum of erythrocyte's hemoglobin before (control) and after 30 min of AMF exposure. The spectra are normalized to the maximum intensity of the peak at 1580 cm^{-1}.

2.3 Statistical Analysis

The *t*-test was used to assess the statistical significance of the results. The results were considered significantly different at $p < 0.05$. The number of measurements was 3.

3 Results and Discussion

3.1 Changes in the Oxygen-Transport Function of Erythrocytes after Exposure to AMF

It is hypothesized that blood cellular elements may significantly influence the changes in the OTF during *in vitro* AMF exposure. Therefore, to assess the influence of AMF on the OTF of rat erythrocytes,

Table 1 Effect of the oxygen affinity of Hb (M ± m) following AMF exposure to rat erythrocytes *in vitro* (* − $p < 0.05$ relative to the control (without exposure to AMF)).

Parameters	Exposure AMF			
	Addition of 0.9% NaCl isotonic solution		Addition of plasma	
	Control	Erythrocytes	Control	Erythrocytes
$p50_{real}$, mm Hg	42.8 ± 2.9	50.7 ± 1.7*	41.1 ± 1.4	46.2 ± 0.8*
$p50_{stand}$, mm Hg	35.1 ± 2.04	39.8 ± 1.01*	38.2 ± 1.16	38.9 ± 1.52*

AMF – alternating magnetic field.

Table 2 Changes in nitrate/nitrite content after AMF exposure in rat blood *in vitro* (M ± m) (* − $p < 0.05$ relative to control (without exposure to AMF)).

Parameters	Control	AMF exposure time	
		120 s	360 s
nitrate/nitrites, μmol	7.32 ± 0.34	8.61 ± 0.41*	9.26 ± 0.4*

AMF – alternating magnetic field.

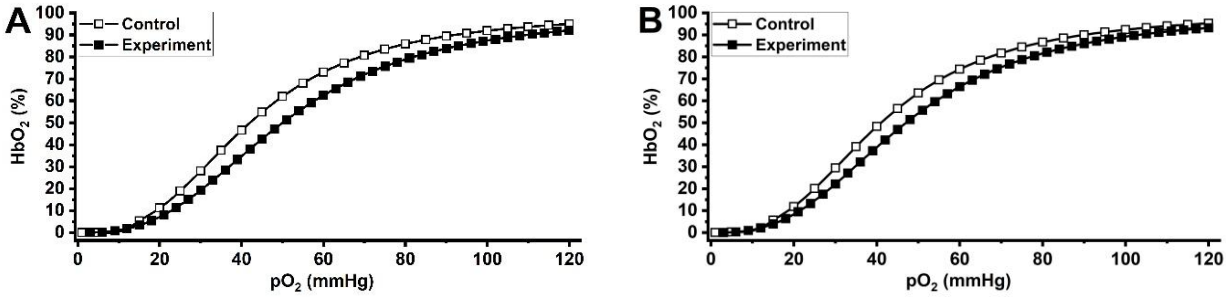

Fig. 2 The effect of AMF exposure on the position of the oxyhemoglobin dissociation curve in (A) erythrocytes with the addition of isotonic 0.9% NaCl solution and in (B) erythrocytes with the addition of plasma.

mixtures of erythrocytes with an isotonic 0.9% NaCl solution and erythrocytes with plasma depleted of leukocytes and platelets were prepared (Table 1, Fig. 2). After exposing *in vitro* the erythrocyte samples to AMF for 120 s, we found that the value of the $p50_{real}$ parameter increased more markedly in the erythrocytes mixture with the addition of the isotonic 0.9% NaCl solution-by 33.18% ($p < 0.05$)-compared to the erythrocytes mixture with added plasma (where the increase in $p50_{real}$ was 12.41% ($p < 0.05$)). This indicates a rightward shift of the oxyhemoglobin dissociation curve under the influence of AMF. The $p50_{stand}$ value also increased in the erythrocytes with the isotonic 0.9% NaCl solution by 13.39% ($p < 0.05$).

The production of the gasotransmitter NO was assessed by the content of nitrate/nitrites in blood plasma (Table 2). At the 120-s mark, this indicator increased by 17.62% ($p < 0.01$), and a further increase in exposure time was accompanied by a rise in NO concentration by 26.5% relative to the control ($p < 0.01$) at 360 s of exposure.

Thus, we have demonstrated that the AMF exposure for 360 s causes a modification of the gasotransmitter producing function. These changes may significantly influence the alteration of the blood's OTF and, consequently, the adequate provision of tissue oxygen demands by blood flow [16]. The obtained results indicate the lack of contribution from blood cellular elements leukocytes and platelets to the change in OTF and the involvement of the gasotransmitter-producing function in modifying the blood's OTF.

3.2 Changes in Hb Conformation after Exposure to AMF

Changes in the conformation of the heme and the packing density of the globin amino acids in isolated and cytoplasmic Hb under the exposure of AMF were assessed in human erythrocytes with measurements taken every minute for 30 min. To analyze the conformational changes, the following ratios of characteristic Raman bands for Hb molecule after AMF exposure and without AMF exposure were analyzed (Fig. 3). We have demonstrated that the dynamics of changes in the ratios of Raman spectrum band intensities in the absence of AMF exposure do not show

statistically significant changes relative to the onset of changes. Conformational changes in isolated erythrocytes may be caused by gradual oxygenation of the erythrocytes and the thermal effect of the laser (~0.03 J in 10 s). At the same time, erythrocytes during their vital activity (NO synthesis, decrease in ATP, glucose) can extract oxygen from the buffer, which also affects the conformation of the heme. However, the dynamics of changes in the absence and with AMF exposure differ. The observed conformational changes during the measurement of isolated Hb are likely caused by reversible photodissociation of the Fe-O bond [23]. Therefore, it can be concluded that the observed changes with AMF exposure are caused by the magnetic field, not the thermal effect of the laser.

Notably, the groups of isolated Hb and cytoplasmic Hb (Hb_b) without and with AMF exposure, showed different patterns relative to the start of the experiment. Thus, changes in the conformation of Hb are caused by the action of AMF.

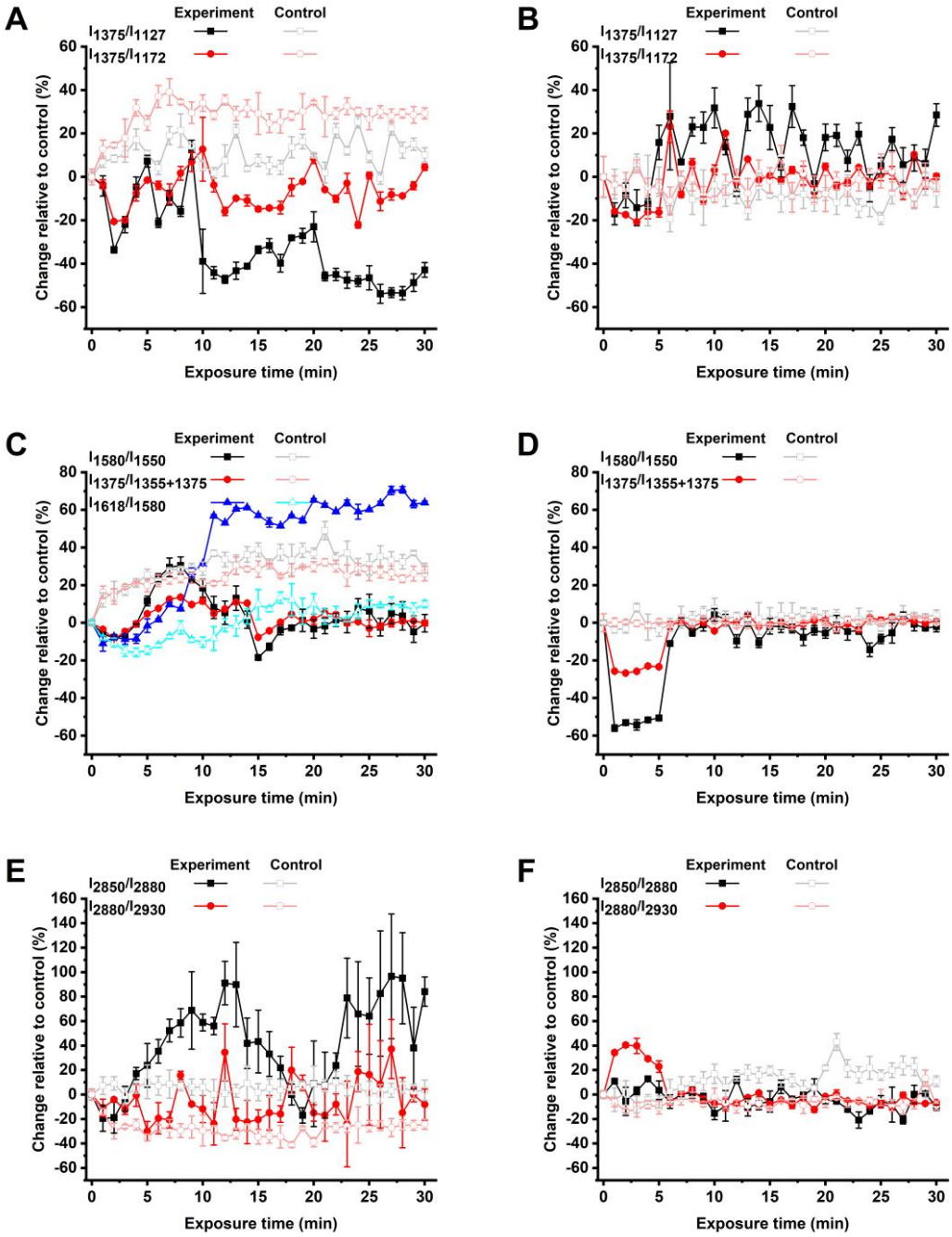

Fig. 3 Changes in the intensity ratio of the Raman peaks for (A, C, E) Hb in the erythrocyte cytoplasm (Hb_b) and for (B, D, F) isolated Hb in phosphate buffer (*in vitro*). (A–D) panels demonstrate changes in heme conformation, (E–F) panels demonstrate changes in conformation of globin. The data are expressed as a change relative to the baseline (0 min). Open circles demonstrate the ratio values for the control group (without AMF exposure). The data are normalized to the beginning of the measurements without the influence of AMF.

We have established that the effects of AMF on the conformation and packing density of isolated Hb and cytoplasmic Hb are manifested differently. This may be due to both the oligomerization of Hb molecules inside the erythrocyte and the biochemical processes within the cell in response to AMF exposure. Specifically, changes in the conformation of isolated Hb were most pronounced in the first 5 min of PMF exposure (Fig. 3, (B, D, F)): an increase in globin packing density is accompanied by a decrease in the proportion of oxyhemoglobin in the sample, indicating a decrease in the affinity of Hb for oxygen. Probably, AMF affects the mobility of heme (an increase in the I_{1375}/I_{1127} ratio), which leads to an increase in the proportion of oHb in the sample to the initial values (without the influence of AMF) from the 5th min of measurements.

For the cytoplasmic Hb, the most pronounced changes in heme conformation are observed during the first 12 min of exposure to an AMF (Fig. 3 (A, C, E)): the proportion of Hb with a planar heme conformation increases, ligand affinity rises (increase in I_{1580}/I_{1550}, $I_{1375}/I_{(1355+1375)}$), and the packing density of globin increases (I_{2880}/I_{2930}, I_{2850}/I_{2880}), indicating an increase in the fraction of oHb in the sample. Considering that the changes in Hb conformation are accompanied by an increase in the number of Hb complexes with NO (a 60% increase in I_{1618}/I_{1580}, $p < 0.05$), the AMF appears to influence the activation of the NO synthase enzyme inside erythrocytes. Subsequently, when the content of Hb-NO complexes reaches a steady-state level, a decrease in ligand affinity and an increase in the proportion of Hb with a dome-shaped heme conformation by 10–12% relative to the control were detected. Thus, changes in the cytoplasm (ordering of Hb molecules, alteration of their conformation, and possible redistribution within the erythrocyte cytoplasm [19–21]) lead to changes in the content of total nitrates/nitrites and their complexes in the erythrocyte cytoplasm.

Increasing the AMF exposure time to 15–30 min, changes in the valence vibrations of the pyrrole rings (decrease in I_{1375}/I_{1172}) and an increase in the packing density of globin (I_{2880}/I_{2930}) were revealed. The observed changes in globin packing density may indicate an alteration in the membrane surface charge and the binding of Hb molecules to band 3 protein (AE1) [24].

The experiment demonstrated that the exposure to AMF for 30 min is accompanied by a non-monotonic change in heme conformation and globin packing density. Depending on the exposure duration, the OTF of erythrocytes is influenced by the activity of NO synthase, which leads to increased binding of NO by Hb molecules in erythrocytes upon AMF exposure. Another contributing factor is the conformational changes of the heme. We hypothesize that with AMF exposure times exceeding 15 min, the decrease in hemoglobin's oxygen affinity may be due to a changes in the spin state of Fe [25, 26].

4 Conclusions

In this study, we demonstrated that exposure to AMF for 30 min significantly affects not only the OTF of erythrocytes and isolated Hb but also the mobility of the globin. Exposure to AMF is accompanied by an increase in NO_3^-/NO_2^- levels and a decrease in hemoglobin's affinity for oxygen, suggesting that AMF-induced changes in OTF are primarily mediated by the gasotransmitter nitric oxide.

Exposure to AMF for 30 min leads to an increased proportion of Hb with the T- state heme and enhanced affinity for ligands (oxygen and NO) (I_{1375}/I_{1127}, I_{1580}/I_{1550}), decrease in the proportion of oHb in the sample (changes in the ratio of Raman scattering band intensities $I_{1375}/I_{(1355+1375)}$. AMF has a significant effect on increasing the packing density of CH-groups of globin amino acids of intracellular Hb (I_{2850}/I_{2930}, I_{2880}/I_{2930}), which may affect the efficiency of OTF. Moreover, the effects of AMF are more pronounced in Hb of erythrocytes than in isolated Hb and do not depend on the presence of other blood cellular elements (leukocytes, platelets).

Conflicts of Interest

The authors declare that they have no conflicts of interest or competing financial interests related to this work.

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