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New view on the compatibility of hemoglobin function in the erythrocytes

Vladyslav V. Smiianov[1], Tetiana V. Fartushok[2], Yuri M. Fedevych[2], Nadiia V. Fartushok[3]
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ABSTRACT

Aim: To study the process of hemoglobin oxidation and the enzymatic reactions associated with it.

Materials and Methods: Heparinized human blood (15 IU/ml) was obtained from the clinical department. The concentration of oxy- and methemoglobin, auto-oxidation of hemoglobin was determined spectrophotometrically spectrophotometrically. Autooxidation of hemoglobin was recorded spectrophotometrically, and protein concentration was determined by the Lowry method. Monooxygenase activity of hemoglobin was also measured by the method described by Lowry spectrophotometrically. The concentration of O_2 and H_2O_2 in the reaction media was determined on a biomicroanalyzer OR 210/3 (Redelkis).

Results: The obtained experimental data allow us to propose a mechanism of "spontaneous autooxidation" of oxyhemoglobin, which can be described by the following equations:

$Hb^{2+}O_2 \longrightarrow Hb^{3+} + O_2^-$ (1)

$Hb^{2+}O_2 + 2e^- + 2H^+ \longrightarrow Hb^{3+} + H_2O_2$ (2)

$Hb^{2+}O_2 + 2e^- + 2H^+ \longrightarrow Hb^{2+} + H_2O_2$ (3)

$Hb^{2+} + O_2 \longrightarrow Hb^{2+}O_2$ (4)

Spectral characteristics of the process of "spontaneous auto-oxidation" indicate the formation of a metform of hemoglobin, the depletion of oxygen by the system was established, at pH 5.6, an increase in the monooxygenase activity of hemoglobin is observed 3-4 times compared to the physiological level.

Conclusions: In addition to the main, previously known functions of hemoglobin (gas transport, peroxidase, monooxygenase), it catalyzes a two-electron oxidase reaction in which O_2 is reduced to H_2O_2. This is confirmed by experimental data on the formation of one of the products of "spontaneous autoxidation" of oxyhemoglobin _ deoxyform at pH 5.6 _ 8.9.

KEY WORDS: oxyhemoglobin, methemoglobin, autooxidation, monooxygenase activity, oxidase reaction, ligands

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INTRODUCTION

The hemoglobin molecule in erythrocytes can undergo oxidation (autooxidation) or be oxidized by other factors, losing the ability to carry oxygen. The concentration of methemoglobin (metHb) in a healthy person usually does not exceed 1% of the total amount of hemoglobin. This level of methemoglobin is the result of a balance between methemoglobin formation and its recovery. In patients with diaphorase I (methemoglobin reductase) deficiency, about 3% of total hemoglobin is oxidized every day [l-3].

The reversibility of the binding of the ligands of the prosthetic group of hemoglobin is due to a protein component that forms a specific environment for heme, which in turn prevents its oxidation. However, subtle exchange of ligands (O2, 2,3-DFH, NO) would be impossible in the presence of rigid protection of the prostaglandin group. Ligands reach the active center of hemoglobin as a result of coordinated small-scale fluctuations of certain side groups of amino acid residues of the globule (β-93), which ensure the reduction of the internal molecular barrier and the formation of the trajectory of the ligand in the direction of heme [4]. It should be noted that in conditions of cessation of oxygen access from the external environment, oxyhemoglobin is capable of autooxidation due to oxygen, which is bound to heme iron [5].

The analysis of literature data allows us to conclude that the exact mechanism of spontaneous oxidation is unknown, and the explanations that exist today are quite contradictory [5]. None of the known works present data on the formation of reduced hemoglobin, which under certain conditions is a transitional form in the process of spontaneous autoxidation of its

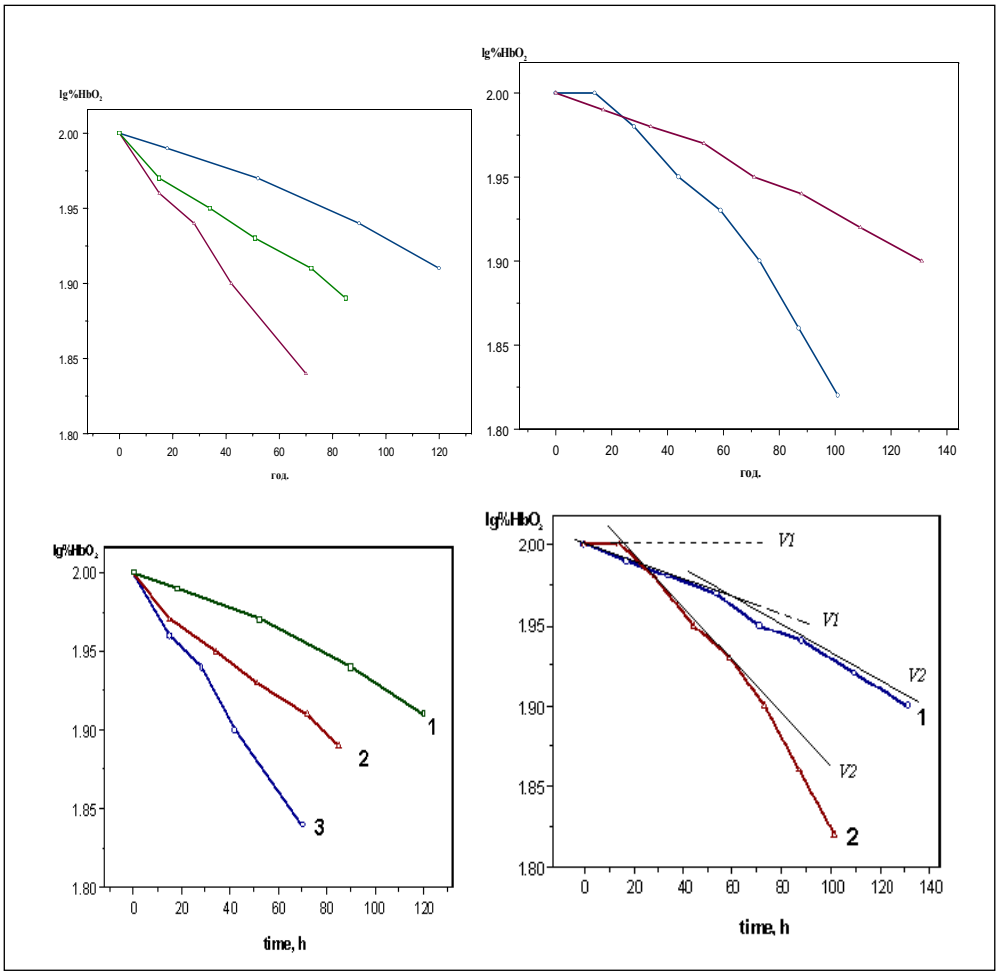

Fig. 1A. Kinetics of the spontaneous autooxidation of the human hemoglobin.
Reaction medium (3,0 ml 0,1 M acetate buffer pH 5,6) contained 0,001M EDTA and 50 – 60 μmoll oxyhemoglobin. 1. – oxyhemoglobin not purified from low-molecular ligands; 2 – oxyhemoglobin purified from low-molecular ligands; 3 – oxyhemoglobin purified from low-molecular ligands and 2,3-DPG. Temperature of reaction medium - 37°C. n = 5, p< 0,05;
B. Kinetics of the spontaneous autooxidation of the human hemoglobin. Reaction medium (3,0 ml 0,05 M tris-HCl buffer pH 7,2) contained 0,001M EDTA and 50 – 60 μmoll oxyhemoglobin. 1. – oxyhemoglobin not purified from low-molecular ligands; 2 – oxyhemoglobin purified from low-molecular ligands. Temperature of reaction medium - 37°C. n = 5, p< 0,05.

prosthetic group. In connection with the above, the study of the process of hemoglobin oxidation and the enzymatic reactions associated with it is relevant today.

AIM

The purpose of our research is to study the process of hemoglobin oxidation and the enzymatic reactions associated with it.

MATERIALS AND METHODS

Experiments were conducted with human hemoglobin, which was isolated from peripheral blood and purified [6, 7]. Heparinized human blood (15 IU/ml) was obtained from the clinical department of Danylo Halytskyi Lviv National Medical University. The concentration of oxy- and methemoglobin was determined spectrophotometrically [8]. Autooxidation of hemoglobin was recorded spectrophotometrically [2], and protein concentration was determined by the Lowry method. Monooxygenase activity of hemoglobin was also measured by the method described by Lowry spectrophotometrically [7]. The concentration of O_2 and H_2O_2 in the reaction media was determined on a biomicroanalyzer OR 210/3 (Redelkis).

RESULTS

The detected products of the "spontaneous autoxidation" reaction indicate the previously not fully understood and unknown properties of hemoglobin. Thus, oxyhemoglobin is able to "spontaneously autoxidize" into metform through a reaction, the speed of which depends on the degree of its purification from low molecular weight compounds (Fig. 1A). With an increase in pH, that is, at physiological values, the kinetics of the rate of "spontaneous autoxidation" of oxyhemoglobin has a complex nature and is characterized by rates V1 and V2 (Fig. 1B). For the first time, it was shown spectrophotometrically that at physiological pH values, when "spontaneous autoxidation" occurs, in addition to metform, the deoxyform of hemoglobin is also formed. This is evidenced by the absorption spectra of hemoglobin after depressurization of the cuvettes (Fig. 2A, B).

As can be seen, low-molecular compounds on the one hand activate "spontaneous auto-oxidation", and on the other - protect oxyhemoglobin from oxidation to met-

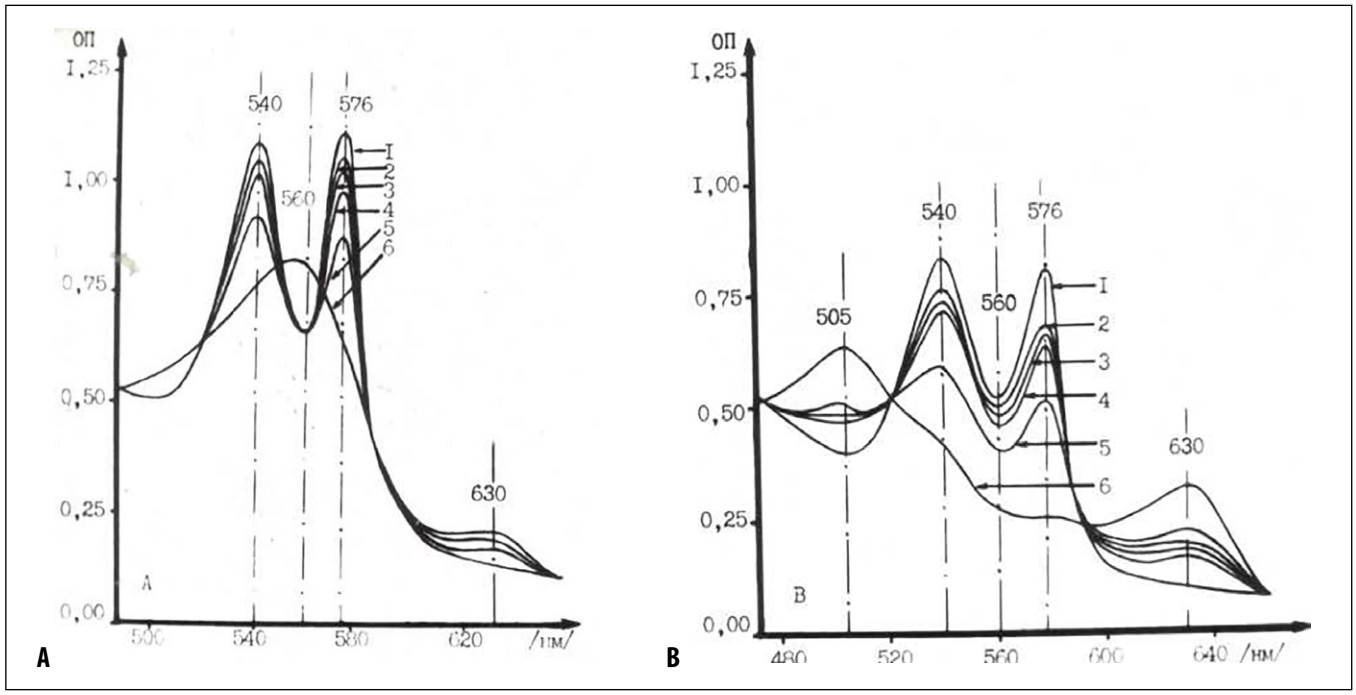

Fig. 2. A. Specters of absorbtion of hemoglobin with low-molecular ligands. Spectra were obtained after 1 _ 24 hours, 2 _ 48 hours, 3 _ 72 hours; 4 _ 96 hours, 5 _ 120 hours, 6 _ 144 hours;
B. Specters of absorbtion of hemoglobin purified from low-molecular ligands. Spectra were obtained after 1 _ 24 hours, 2 _ 48 hours, 3 _ 72 hours; 4 _ 96 hours, 5 _ 120 hours, 6 _ 144 hours

form. Therefore, low-molecular-weight compounds at physiological pH values take a direct part in stabilizing the conformational state of the hemoglobin molecule, that is, stimulating its "spontaneous auto-oxidation", while not changing the oxidation-reduction state of iron in the heme structure. This is not observed when pH decreases, in particular at pH 5.6.

In addition to the already known, such a protective system is the ingredient low-molecular composition of the erythrocyte. Thus, at physiological pH, complete oxidation of oxyhemoglobin to metform does not occur, and after depressurization of the cuvettes, oxygenation of deoxyhemoglobin is observed. Hemoglobin, which contains low-molecular-weight compounds, is oxygenated faster compared to purified hemoglobin (Fig. 2A).

The obtained experimental data, as well as information from the literature [8], allow us to propose a mechanism of "spontaneous autoxidation" of oxyhemoglobin, which can be described by the following equations:

$Hb^{2+}O_2 \longrightarrow Hb^{3+} + O_2^-$ (1)

$Hb^{2+}O_2 + 2e^- + 2H^+ \longrightarrow Hb^{3+} + H_2O_2$ (2)

$Hb^{2+}O_2 + 2e^- + 2H^+ \longrightarrow Hb^{2+} + H_2O_2$ (3)

$Hb^{2+} + O_2 \longrightarrow Hb^{2+}O_2$ (4)

During hemolysis of erythrocytes (extracellular) hemoglobin can enter the bloodstream, where the concentration of antioxidant enzymes is low, and act as a source of iron ions, which is not active in oxyhemoglobin. Hydrogen peroxide, hypochlorous acid, organic lipid hydroperoxides can modify oxyhemoglobin with the formation of more reactive compounds _ feryl and perferrylhemoglobin [23]. Hemoglobin modification products by oxidants are able to cause peroxidation of lipids, blood lipoproteins, lipids of biomembranes [24] and other important biomolecules of the body [25], thus being the cause of the occurrence and development of various diseases.

Mechanisms of cellular and extracellular hemoglobin oxidation, as well as the influence of factors on cellular and extracellular "spontaneous autooxidation" of hemoglobin are presented in Fig 3 and Fig 4.

At a pH value of 5.6, reactions 1-4 occur, because, firstly, the spectral characteristics of the process of "spontaneous autoxidation" indicate the formation of the metform of hemoglobin (reactions 1, 2), and secondly, it is established that the system is depleted of oxygen (reactions 3, 4), thirdly, at this pH there is an increase in the monooxygenase activity of hemoglobin by 3-4 times compared to the physiological level.

DISCUSSION

Blood hemoglobin is always at risk of oxidation to methemoglobin, in which the molecule retains its original structure but can no longer carry oxygen.

It is believed that part of the oxygenated hemoglobin HbFe2+O2 is located in the form of $HbFe^{3+}O_2$-. [4]:

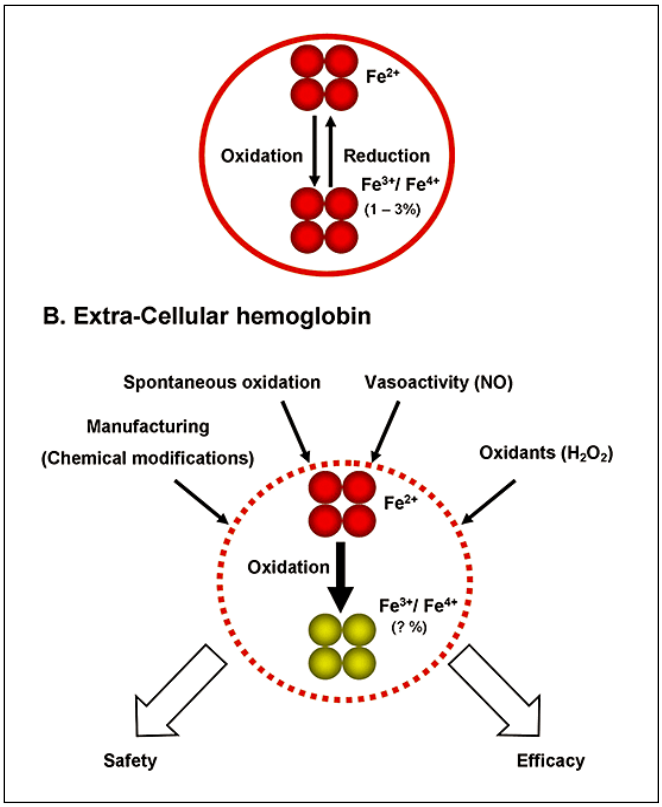

Fig. 3. Factors influencing cellular and acellular hemoglobin oxidation. The schematic represents reductive processes of (A) cellular and (B) extra-cellular hemoglobin. In (A) the oxidation followed by enzymatic and non enzymatic processes in the red blood cell lead to minimal accumulation of $HbFe^{3-}$and $HbFe^{4+}$. The physical barrier of the red cell membrane (red circle) limits NO and H_2O_2 mediated oxidative processes. In (B) when the protective mechanisms of the red blood cell are eliminated such as is the case with HBOCs or when Hb is released, the influences of enzymatic/non-enzymatic reductive processes are lost allowing for an unknown amount of oxidized hemoglobin accumulation. Moreover each arrow indicates processes, which may directly lead to the accumulation of oxidized Hb.

$HbFe^{2+}O_2 \leftrightarrow HbFe^{3+}\ O_2$-.(1)

(the initial stage of autoxidation corresponds to the dissociation of this form into methemoglobin ($HbFe^{3+}$) and O_{2-} (superoxide anion):

$HbFe^{3+}O_2^{-\cdot} \rightarrow HbFe^{3+} + O_2^{-\cdot}$ (2) [1, 4, 5]

formed by O2-. attacks the new oxyhemoglobin molecule in the presence of H+ ions (low pH):

$HbFe^{2+}O_2 + O_2^{-\cdot} + 2H^+ \rightarrow HbFe^{3+} + H_2O_2 + O_2$ (3)

The resulting H_2O_2 [3] attacks the new oxyhemoglobin molecule:

$HbFe^{2+}O_2 + H_2O_2 \rightarrow HbFe^{3+}\ OH\cdot + OH^- + O_2$ (4)

OH the radical that is formed from oxyhemoglobin can then react with other oxyhemoglobin molecules:

$HbFe^{2+}O_2 + OH\cdot \rightarrow HbFe^{3+} + 2OH^- + O_2$ (5)

Peroxides react with oxyhemoglobin $HbFe^{+}O_2$ or methemoglobin with formation of protein-associated oxidant type ($HbFe^{4+}$) and dangerous peroxide radicals [6, 7, 8-13].

$HbFe^{2+}O_2 + H_2O_2 \rightarrow HbFe^{4+}OH + OH\cdot + O_2$ with formation O_2 (5)

$HbFe^{3+} + H_2O_2 + H^+ \rightarrow HbFe^{4+}OH + H_2O$ (6)

In erythrocytes, this highly toxic protein radical reacts with $HbFe^{2+}O_2$:

$HbFe^{4+} + HbFe^{2+}O_2 \rightarrow 2HbFe^{3+} + O_2$ (7)

The formed methemoglobin is restored (reduced) by methemoglobin reductase [14-18].

In addition to self-oxidation, hemoglobin can be oxidized by the superoxide radical (O^{2-}), which is constantly generated in erythrocytes [l] and in all aerobic cells [16]:

$O_2 + e^- \rightarrow O_2^{-\cdot}$(8)

This radical can affect the oxidation of oxyhemoglobin to methemoglobin [4].

Hemoglobin can be oxidized by nitric oxide, which is produced by endothelial cells and released into the

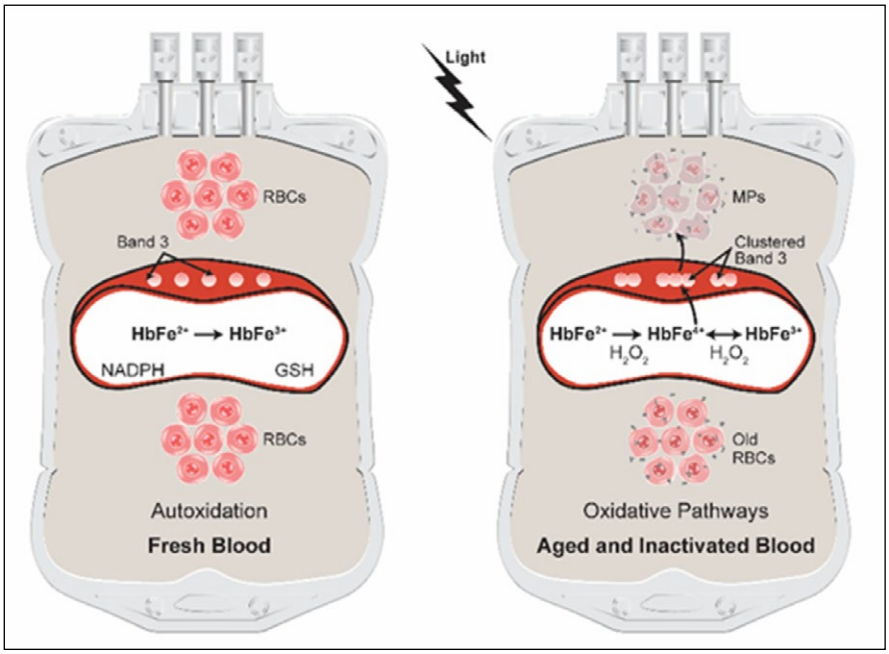

Fig. 4. Oxidative damage of erythrocytes during storage and conditions of pathogen inactivation. Freshly stored RBCs in a standard blood bag undergo very little oxidation apart from normal spontaneous (autoxidation) reactions of Hb, resulting in little metHb accumulation (left). Reductive and antioxidant enzymes/proteins such as NADPH reductase and GSH maintain metHb to a minimum. Under prolonged storage conditions or when RBCs are exposed to UV light, Hb oxidative side reactions are increased, mainly Hb's own pseudoperoxidative pathways (right). These pathways result in the production of ferryl Hb ($HbFe^{4+}$) which attacks other biological targets including band 3, resulting in band 3 clustering. Ferryl Hb crosslinks the major RBC membranes band 3 into clusters and the ultimate release of Hb-laden microparticles (MPs), based on with modifications.

Table 1. Antioxidant enzymes and concentrations of reducing agents in human plasma and erythrocytes [17].

Concentration	Plasma	Erythrocytes
Antioxidant Enzymes	(U ml^{-1})	(U-10^{-10} cells)
Superoxide dismutase	5-20	550-800
Catalase	?	3800-5400
(GSH) peroxydase	0.4	7.8 -10.6
(GSSG) reductase	0.0	2.7-3.7
Reducing Agents	μM	μM
Glutathione (GSH)	5	2.5 $l0^3$ to 10 $l0^3$
Ascorbic acid	45 to 85	40 to 70

bloodstream, and can enter the erythrocyte. Thus, hemoglobin will probably be oxidized as follows [4]:

$2HbFe^{2+}O_2 + 2{\cdot}NO + e^- \rightarrow 2HbFe^{3+} + 2NO_2{\cdot} + O_2^{-}{\cdot}$ (9)

or by the equation:

$2{\cdot}NO + O_2 \rightarrow 2{\cdot}NO_2$ (12) [24]:

$HbFe^{2+}O_2 + {\cdot}NO_2 \rightarrow HbFe^{3+} + NO_2^- + O_2$ (10)

As a result of the reaction, the nitrite anion NO_2- is formed, which reacts with a new molecule of oxyhemoglobin in an acidic environment:

$HbFe^{2+}O_2 + 2H^+ + NO_2^- \rightarrow HbFe^{3+} + {\cdot}NO_2 + H_2O_2$ (11) [19]

or by the equation:

$HbFe^{2+}O_2 + {\cdot}NO \rightarrow HbFe^{3+} + NO_3^-$ (15) [20].

H_2O_2 and O_2- are formed. can react with a new molecule of oxyhemoglobin according to reactions 3-4. Hemoglobin in erythrocytes or in blood plasma would not perform the function of oxygen transfer if there was no enzymatic system to inhibit its oxidation reactions.

Reactions limiting hemoglobin oxidation [21].

Since superoxide anion and hydrogen peroxide are naturally formed in the body, there are enzyme systems that protect hemoglobin from oxidation: superoxide dismutase to neutralize superoxide anion (O_2^-), glutathione peroxidase and catalase to split H_2O_2 [5, 13, 22]. The activity of these enzymes is high in erythrocytes and low in blood plasma [23].

Erythrocytes contain enzymatic or non-enzymatic systems that restore the hemoglobin molecule to its active form and physiological function, keeping the level of circulating methemoglobin below 1%. Methhemoglobin recovery pathways are mainly enzymatic and associated with erythrocyte glycolysis (Table 1).

Superoxide dismutase (SOD) catalyzes the reaction:

Superoxide dismutase

$2O_2^{-}{\cdot} + 2H^+ \rightarrow H_2O_2{\cdot} + O_2$ (12)

Low concentrations of H2O2 are mainly decomposed by glutathione peroxidase [18, 24]:

Glutationperoxidase

$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$ (13)

Then, as the decomposition of high concentrations of H2O2 is catalyzed by catalase:

Catalase

$H_2O_2 \rightarrow H_2O + ½\ O_2$ (14)

These reactions depend mainly on hydrogen sources, such as NAD and NADPH [23, 26, 27].

An important supplier of hydrogen is the NAD-dependent metabolic pathway associated with anaerobic glycolysis. NAD is reduced to NADH, which is then used by methemoglobin reductase (diaphorase I, NADH dehydrogenase, or cytochrome b5 reductase [25, 27].

Cytochrome b5-reductase

$NADH + Cytb_5Fe^{3+} \rightarrow NAD^+ + Cytb_5Fe^{2+}$ (15)

$Cytb_5Fe^{2+} + HbFe^{3+} \rightarrow Cytb_5Fe^{3+} + HbFe^{2+}$ (16)

One molecule of cytochrome b5 can bind to one subunit of methemoglobin, mainly through lysine and/or arginine residues [27], and transfer the electron needed to reduce iron to the divalent state.

Another supplier of hydrogen is NADP, the main place of its formation is the pentose phosphate pathway of glucose metabolism. NADPH is reduced to NADPH, which itself reduces methemoglobin to hemoglobin under the action of another methemoglobin reductase, NADPH-flavin reductase, diaphorase II or NADPH-dehydrogenase [28, 29]:

NADPH- reductase

NADPH + flavin *(oxidized form)* $\rightarrow$ $NADP^+$ + dihydroflavin *(reduced form)* (22)

dihydroflavin + $Fe^{3+} \rightarrow$ flavin + Fe^{2+} (17).

In a non-enzymatic way, methemoglobin is reduced to hemoglobin as a result of the reaction with ascorbic acid or reduced glutathione (Table 1).

Ascorbic acid can penetrate the erythrocyte membrane and reduce the level of methemoglobin by transferring one electron

At the same time, ascorbic acid is oxidized to dehydroascorbic acid, which is then reduced by glutathione or directly by dehydroascorbate reductase [30, 31]:

So, the process of auto-oxidation of hemoglobin can be represented by the total equation:

$4\ (HbFe^{2+}\ O_2) + 2H^+ \rightarrow 4HbFe^{3+} + 2OH^- + 3O_2$ with formation $O_2^{-}{\cdot}$, $OH{\cdot}$, H_2O_2 (6)

Thus, gemoglobin (Hb) within red blood cells (RBC) is protected from oxidative processes by efficient enzymatic machineries such as cytochrome *b*5, or flavin, coupled with NADH-dependant or NADPH-dependent methemoglobin reductases, reduced glutathione (GSH) and small molecule reductants which include ascorbic acid and uric acid. Thus when small amounts of Hb are oxidized to ferric ($HbFe^{3+}$) and ferryl ($HbFe^{4+}$) forms, reduction to the oxygen carrying ferrous ($HbFe^{2+}O_2$) form occurs rapidly to restore oxygen carrying capability and prevent cellular injury which may be triggered by these oxidation intermediates (Fig. 3).Moreover, the RBC provides a functional protective barrier to excessive nitrosative agents such as endothelial-derived nitric oxide (NO) and peroxidative agents such as hydrogen peroxide (H_2O_2).

According to literature data, the physiological oxidation of hemoglobin is characterized by a certain sequence of autoxidation and oxidation reactions, as well as the presence of factors in erythrocytes and blood plasma that can reduce the level of methemoglobin or prevent its formation.

CONCLUSIONS

Thus, based on the above data, it can be concluded that in addition to the main, previously known functions of hemoglobin (gas transport, peroxidase, monooxygenase), it catalyzes a two-electron oxidase reaction in which O_2 is reduced to H_2O_2. The validity of this position is confirmed by experimental data on the formation of one of the products of "spontaneous autooxidation" of oxyhemoglobin - deoxyform - THIS is not oxidation of hemoglobin, iron remains +2 as well as the kinetic characteristics of this process in the range of pH 5.6 _8.9. It has been shown that the formation of hemoglobin complexes with low-molecular-weight compounds that are formed in the erythrocyte contributes to the preservation of its functional activity. This property acquires a special physiological significance in conditions of low pO2 values, when the probability of loss of the gas transport function of hemoglobin increases sharply, as a result of possible oxidation of the iron porphyrin structure.

The obtained results can be used for the objective assessment of oxygen binding properties of hemoglobin in patients with distress, destructive processes and intoxication.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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