International Journal of Multidisciplinary Research and Development
www.allsubjectjournal.com
ISSN Online: 2349-4182 ISSN Print: 2349-5979
Received: 02-12-2023, Accepted: 17-12-2023, Published: 02-01-2024
Volume 11, Issue 1, 2024, Page No. 1-5

Concentration dependence of the influence of sodium nitrite on the oxidative degradation of hem during hydrogen peroxide oxidation of hemoglobin

T M Huseynov[1], S M Rahmanova[1], H A Abiyev[2]
[1] Department of Ecological Biophysics, Institute of Biophysics, Ministry of Science and Education of Azerbaijan Republic, Baku, Azerbaijan
[2] Department of Medical and Biological Physics, Azerbaijan Medical University, Baku, Azerbaijan

Abstract
The study examined the concentration dependence of the inhibition of oxidative degradation of heme hemoglobin (Hb) induced by a bolus dose of hydrogen peroxide (H_2O_2) with sodium nitrite ($NaNO_2$). Oxidative degradation of heme caused by H_2O_2 is accompanied by the formation of fluorescent heme breakdown products. And nitrites can inhibit this process, despite the fact that they themselves are capable of oxidizing Hb, depriving it of its ability to transfer O_2, and creating hypoxia and have a toxic effect. It was possible to establish the lower concentration limit of the inhibitory effect (NO_2), which starts from 0.5 mM and higher according to the quenching of characteristic fluorescence ($\lambda_{excitation}$ = 321 nm (313 nm), $\lambda_{emission}$ = 465 nm (460 nm) and $\lambda_{excitation}$ = 437 nm, $\lambda_{emission}$ = 520 nm) The effect of maximum inhibition is achieved at concentrations of 2.0 mM-5.0 mM.

Keywords: $O_2^{\cdot-}$, H_2O_2, $NaNO_2$, nitrite ion, fluorescence, hemoglobin, heme

Introduction

The process of interaction of hemoglobin with oxygen is complex and includes: changes in the structure of heme (changes in spin characteristics, modification of the porphyrin ring (unit), up to the release of iron ions, etc.), structural changes in globin subunits (oxidation of SH groups and etc.), violation of cooperativity between subunits [1]. There is growing evidence supporting the role of heme-dependent reactions as major inducers of oxidative damage. In particular, the oxidation of oxyhemoglobin is accompanied by the release (or release) of oxygen with the formation of the superoxide radical $O_2^{\cdot-}$, which dismutates into hydrogen peroxide (H_2O_2) [2, 3, 4, 5]. This triggers a cascade of free radical oxidative modifications of hemoglobin, affecting heme status. And this ultimately leads to the oxidative degradation of heme, associated with the transition from a tetragonal structure to an orthorhombic one, which gives rise to the decomposition of the heme structure [4, 5, 6]. This process is accompanied by the accumulation of two pigment compounds that fluoresce in wide spectral regions. Here, 2 types of connections are defined: excitation $_{max}\lambda_{ex}$ = 321 nm and emission $_{max}\lambda_{exp}$ = 465 nm and λ_{ex} = 460 nm, λ_{emis} = 525 nm [7, 8].

Nitrites, like a number of other active substances (CO, cyanides, etc.), which have a high affinity for hemoglobin, are able to prevent further oxidation of hemoglobin, which could ultimately lead to a conformational rearrangement and destruction of heme and dissociation of the iron ion from the porphyrin ring (cycle) [6, 8, 9].

Inhibition of oxidative reactions in hemoglobin by nitrite can be carried out due to the interaction of nitric oxide (NO.), one of the main metabolites of nitrites, with hemoglobin, which inhibits the Fenton reaction due to the binding of iron ion (Fe^{++}) [10]. And this leads to premature cessation of the formation of the hydroxyl anion (OH^-), which is a strong accelerator of the oxidative reaction [10, 11]. In addition, the main product of nitrite oxidation is -MetHb has certain peroxidase activity [12]. All this, depending on the conditions, "helps" it act as an antioxidant. In this regard, it is of interest to find the concentration limits (within concentrations close to toxic) of the inhibitory effect of nitrites on the oxidative destruction of heme induced by hydrogen peroxide, by weakening the fluorescence intensity.

Materials and methods

Reagents: Iodoacetamide (manufactured by Sigma Aldrich), sodium nitrite ($NaNO_2$), sodium azide, EDTA, potassium phosphate monosubstituted, sodium chloride, 30% hydrogen peroxide (H_2O_2) (ReaKhim Russia) chemical grade.

Erythrocyte mass was isolated from donor blood by 3-fold centrifugation (800g x 10 minutes), which was washed three times with 5 volumes of isotonic solution (0.14 M NaCl). A suspension of erythrocytes (hematocrit (Ht) ≈ 10%) was prepared from it by diluting it with buffer (0.05 M potassium phosphate buffer (PPB) + 0.14 M NaCl, 0.1 mM EDTA, pH = 7.4) for subsequent preparations corresponding aliquots containing ≈50 μM Hb. The study of the inhibitory effect of sodium nitrite ($NaNO_2$) on the oxidative modification of hemoglobin, or its heme, was carried out using final concentrations (FC) in the incubation medium in a wide range of doses from 0.01 mM to 5.00 mM. Incubation time is up to 60 minutes.

The research included 2 series of experiments: 1) control and 2) experimental.

1) In the first series (control), the kinetic dependence of oxidative modification (heme degradation) on H_2O_2 FC in an incubation medium containing erythrocytes was studied based on the increase in fluorescence intensity. Establishment of conditional bolus doses of H_2O_2

2) In the second series (experiment), the effect of $NaNO_2$ FC on the state of oxidative modification of hemoglobin heme exposed to H_2O_2 in a bolus dose was studied.

Establishment of the threshold FC of $NaNO_2$ inhibition by oxidative degradation of heme.

The depth of oxidative modification of hemoglobin (depletion of HbO_2, accumulation of MetHb) was assessed spectrophotometrically (SF-46, Russia) using semi-empirical formulas proposed by Winterbourn [11].

$[oxyHb] = 119A_{577} - 39A_{630} - 89A_{560}$

$[MetHb] = 28A_{577} + 307A_{630} - 55A_{560}$

The fluorescence intensity was measured on a laboratory fluorimetric installation assembled on the basis of a FAS-1 fluorimeter (USSR), where an FEU-64 served as a photodetector and an SVD-120A mercury lamp was used as a light source. For excitation in luminescence in the band with a maximum of 321 nm and 465 nm, mercury spectral lines of 313 nm and 347 nm were used, which were isolated by appropriate light filters. Emission radiation was passed through narrow-band filters (460 nm and 520 nm).

The results were statistically processed ($n \geq 3$) at a significance level of $p<0.05$.

Results and discussions

Before embarking on experiments to determine the effect of sodium nitrite ($NaNO_2$) on the development of oxidative Degradation of hemoglobin caused by H_2O_2, it was necessary to identify the threshold dependence of fluorescence on the concentration of H_2O_2 in the incubation medium containing erythrocytes.

Here we proceeded from the fact that during the H_2O_2-induced oxidation of heme, a cascade of oxidative reactions occurs, the initial step of which is the formation of highly reactive ferrylhemoglobin. The latter [HbFe (IV) = 0], interacting with the second H_2O_2 molecule, generates $O_2^{-\cdot}$ superoxide in the heme pocket, which attacks heme porphyrin, changing its structure [14], which is accompanied by the appearance of two types of fluorescent compounds [7, 8]. As a result, unstable ferrylhemoglobin is reduced to relatively stable methemoglobin according to the final reaction [6, 14]:

HbFe (II) $O_2 + H_2O_2 \rightarrow$ HbFe (IV)=O+H_2O+ O_2 (1)

HbFe (IV)=O + $H_2O_2 \rightarrow$ HbFe (III)+ H_2O+ O_2 (2)

The accumulation of this product can be indirectly assessed by the depletion of HbO_2 and, accordingly, by the accumulation of methemoglobin. Based on this indicator, one can conditionally estimate the concentration subtoxicity of H_2O_2 according to the criterion of ≈ 30-50% depletion of HbO_2 Fig. 1.

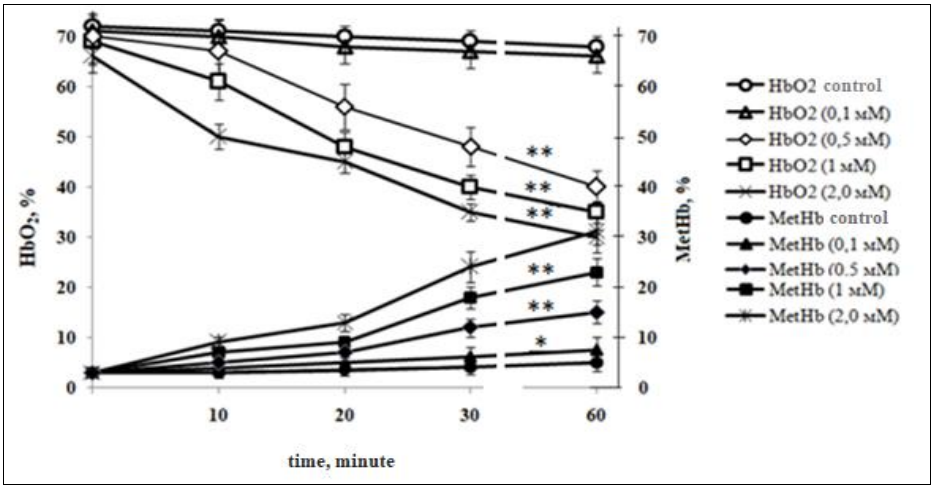

Fig 1: Dependence of the kinetics of H_2O_2-induced hemoglobin oxidation on the concentration of H_2O_2 in the incubation medium (0.05 M PPB 0.14 M NaCl + 0.1 mM EDTA, pH 7.4, t = 37℃), (45 was taken as 100% HbO_2 μM). (* - $p \leq 0.05$; ** - $p \leq 0.01$).

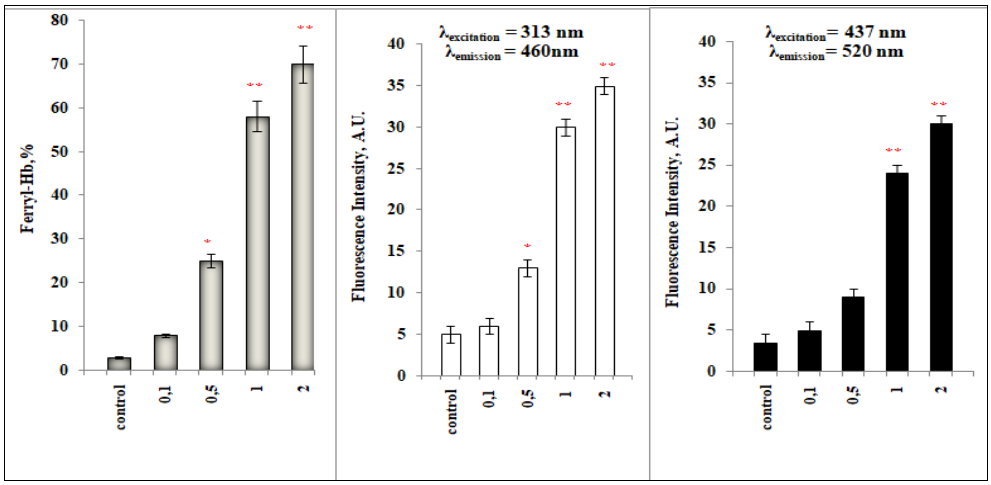

Fig 2: Effect of the final concentration of H_2O_2 in the incubation medium (0.05 M PPB, 0.14 M NaCl + 0.1 mM EDTA, pH 7.4, t = 37˚C, incubation time 60 minutes) containing erythrocytes (≈ 50 μM Hb), on The fluorescenceintensity of heme oxidative degradation products (* - $p \leq 0.05$; ** - $p \leq 0.01$):

a. Accumulation of MetHb in%; take as 100% = 17 µM MetHb;
b. Fluorescence conditions: $\lambda_{excitation}$ = 313 nm, λemission = 460 nm;
c. Fluorescence conditions: $\lambda_{excitation}$ = 437 nm, λemission = 520 nm.

Figure 2 shows that at FC levels of 0.5 mM and higher, accumulation of methemoglobin is also observed, which is reflected in the intensity of the fluorescence peaks of both pigments of heme oxidative degradation. In this case, the increase in the intensity of methemoglobin accumulation is similar to the increase in the intensity of the fluorescence peaks of both pigment compounds. This indicates that the cause of the appearance of fluorescent pigments is the structural rearrangement of heme, which arose during the formation of ferrylhemoglobin during the oxidation of heme with H_2O_2, which is ultimately reduced to MetHb (Fig. 2).
Thus, according to the data in Figure 1 and Figure 2, we can conclude that doses above 0.5 mM H_2O_2 x 30 min. are effective in terms of accumulation of MetHb and it can be conditionally accepted that a dose of 2mM x 30 min. and above are bolus.

In our work, it was necessary to establish subtoxic doses of sodium nitrite that could have an effective inhibitory effect on the oxidative degradation of hemoglobin. For this study, they carried out a wide range of concentrations from $1*10^{-3}$ mM to 5.0 mM, in a nitrite: hemoglobin ratio from 0.1:1.0 to 50:1, i.e. with a concentration range many times higher than physiological [9, 10]. These experiments showed that even a minimal amount ($1*10^{-3}$ mM) already had a noticeable effect on the accumulation of MetHb in erythrocyte suspensions. From Figure 3 shows that during half-hour incubation at 37 ˚ C, MetHb accumulates as quickly as possible in the first 5 minutes, and in the next 5 minutes, the rate of MetHb accumulation decreases significantly. After this period (10 minutes), significant MetHb accumulation occurs for high concentrations (2.0 mM and 5.0 mM) of $NaNO_2$: values up to 70% of the MetHb accumulation level. MetHb accumulation continues up to 100% (Fig. 3) after 20 minutes of incubation, after which it decreases for high concentrations (5.0 mM).

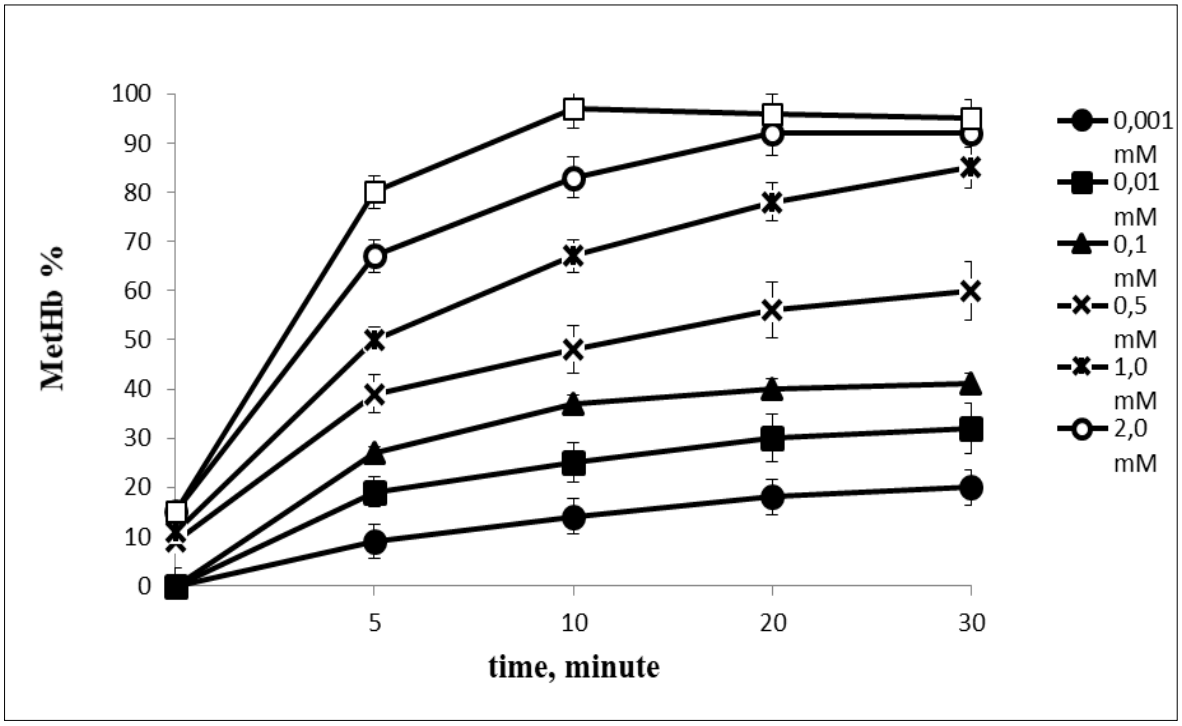

Fig 3: Effect of different concentrations of sodium nitrite on the development of MetHb accumulation in a Suspension (100 µM HbO_2) of erythrocytes. The $NaNO_2$ content was varied to 0.001, 0.01, 0.1, 0.5, 1.0, 2.0 and 5.0 mM. (Incubation conditions are the same as for Figure 1, incubation time 30 minutes).

Thus, taking into account the literature data [9, 10], that when the NO_2:Hb ratio is less than 4:1, the HbO_2 oxidation reaction does not enter the autocatalytic mode and is at the delayed stage (lag status). In our case, concentrations of 0.5-1.0 mM $NaNO_2$ can probably be considered close to toxic, provided that the threshold for the transition of the oxidation reaction to the autocatalytic mode is within the range of NO_2: HbO_2>4. Here the NO_2:Hb ratio is 0.5mM :0.1mM=5 and 1.0mM :0.1mM=10, respectively, which exceeds the critical value.
From the analysis of the data presented in Figure 3, it is clear that the maximum oxidative effect of nitrite occurs in the first 5-10 minutes of incubation.
We therefore pretreated our samples with nitrite for 10 Minutes as an inducer of heme oxidation before adding it to the incubation medium where they would be oxidized by hydrogen peroxide. Here we used concentrations of 0.5 and 1.0 mM $NaNO_2$, which resulted in MetHb accumulation of up to 50-70% within 10 minutes (see Figure 3).
From Figure 4 it can be seen that as the concentration of sodium nitrite in the incubation medium increases, the fluorescence intensity decreases as a result of heme degradation. In this case, we limited ourselves to considering the development of fluorescence with a maximum emission of 460 nm with an excitation of 313 nm. This was due to the fact that the fluorescence products of this band were higher than those of the neighboring (437 nm excitation and 520 nm emission) band.

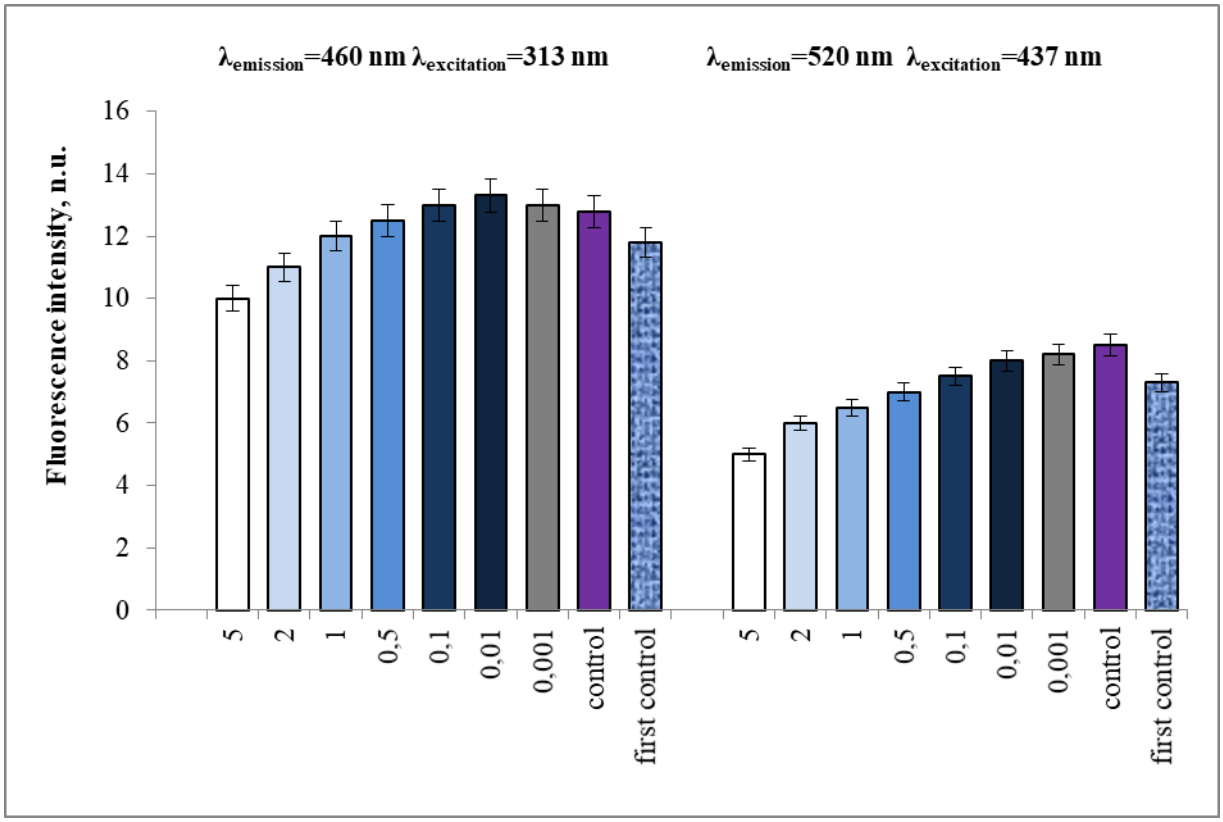

Fig 4: Effect of different concentrations of sodium nitrite on the fluorescence state of hemoglobin. The $NaNO_2$ content was Varied to 0.001, 0.01, 0.1, 0.5, 1, 2 and 5 mm. (Incubation conditions: 0.5 mM PPB, pH 7.4, 37˚C, for 8 hours in the presence of inhibitors: 1 mM sodium azide (SA) and 5 mM iodoacetamide (IA).

Conclusion

Despite their similarity, there are fundamental differences between the oxidation of hemoglobin with nitrites and hydrogen peroxide. The fact is that the main products of nitrite-dependent oxidation are MetHb, MetHb-NO_2, etc. (ferrylhemoglobin is ultimately reduced again to MetHb), which, due to the fact that they are chemically oxidized forms of hemoglobin, have lost the ability to become saturated and carry oxygen [8, 13]. Here, oxygen transport is inhibited, despite the fact that this process is based on the fact that nitrogen oxides have a higher affinity for hemoglobin than oxygen [11, 12]. This means limiting the formation of O_2^- and H_2O_2, since in the absence of O_2 there is no generation of ROS. And this, in the absence of H_2O_2, leads to inhibition of oxidative stress. This suggests that, depending on the concentration, NO_2^- can at certain stages inhibit the development of Hb oxidation, even stopping, especially in its heme part, given that it goes into an autocatalytic mode, thanks to hydrogen peroxide [9, 10, 11].

The ability to quench the fluorescence emitted by the products of oxidative degradation of heme induced by bolus doses of H_2O_2 allows us to evaluate the degree of the AO effect of nitrites. At the same time, this effect is determined by the concentration of nitrite itself. This means that low doses do not have a noticeable effect on fluorescence, but high doses exacerbate the oxidation process as oxidizing agents, forming radical products. That's why, the range of $NaNO_2$ concentrations at which fluorescence is significantly quenched (reduced) must be high enough to have a certain inhibitory effect.

Thus, the toxic effect of nitrites is associated primarily with their oxidative effect, generating methemoglobin, on hemoglobin that has lost the ability to carry oxygen [11]. However, under certain conditions, nitrite, like its metabolite NO (which also determines its toxicity), can also act as an antioxidant [15, 16, 17]. The reason why nitrite-induced MetHb can inhibit the oxidative process is that methemoglobin in the absence of oxygen cannot create its active forms [6, 9, 10]. In addition, methemoglobin has (as a hemeprotein) a certain peroxidase activity similar to catalase [12, 18]. The manifestation of inhibitory properties is de facto associated with stopping or slowing down the oxidative modification (or degradation) of heme, which manifests itself in the form of its ability to quench hydrogen peroxide-induced fluorescence. In our recent studies, we also evaluated the pro- and anti-oxidative significance of nitrite on both hemoglobin and red blood cells in general. Here we rated these frames at 0.35mM and 0.70mM as subtoxic [19]. The fluorescence method, having high sensitivity, can be used for early diagnosis of oxidative damage to hemoglobin.

References

1. Perutz MF, Fermi G, Luisi B, Shaanan B, Liddington RC. Stereochemistry of cooperative mechanisms in hemoglobin. Cold Spring Harbor Symp Quant Biol,1987:52:555.
2. D'Alessandro A, Anastasiadi AT, *et al*. Red blood cell mechanism *in vivo* and *in vitro*. Metabolites,2023:13(7):793.
3. Ahmed MH, Ghatge MS. Hemoglobin: Structure, Function and Allostery. Subcell Biochem,2020:94:345.
4. Möller MN, Orrico F, Villar SF, López AC, Silva N, Donzé M, Thomson L, Denicola A. Oxidants and antioxidants in the redox biochemistry of human red blood cells. ACS Omega,2022:8(1):147.

5. Flohe L. Looking back at the early stages of redox biology. Antioxidants,2020:9(12):1254.
6. Nagababu E, Rifkind JM. Heme degradation by reactive oxygen species. Antioxid Redox Signal,2004:6(6):967.
7. Nagababu E, Rifkind JM. Formation of fluorescent heme degradation products during the oxidation of hemoglobin by hydrogen peroxide. Biochem Biophys Res Commun,1998:247(3):592.
8. Nagababu E, Chrest FJ, Rifkind JM. The origin of red cell fluorescence caused by hydrogen peroxide treatment. Free Radic Biol Med,2000:29(7):659.
9. Keszler A, Piknova B, Schechter AN, Hogg N. The reaction between nitrite and oxyhemoglobin: a mechanistic study. J Biol Chem,2008:283:9615.
10. Piknova B, Keszler A, Hogg N, Schechter AN. The reaction of cell-free oxyhemoglobin with nitrite under physiologically relevant conditions: implications for nitrite-based therapies. Nitric Oxide, 2009:20:88.
11. Owusu BY, Stapley R, Honavar J, Patel RP. Effects of erythrocyte aging on nitric oxide and nitrite metabolism. Antioxid Redox Signal,2013:19(11):1198.
12. Widmer CC, Pereira CP, Gehrig P, Vallelian F, Scoedon G, Buehler PW, Schaer DJ. Hemoglobin can attenuate hydrogen peroxide-induced oxidative stress by acting as an antioxidative peroxidase. Antioxid Redox Signal,2010:12(2):185.
13. Winterbourn CC. Oxidative reactions of hemoglobin. Methods Enzymol, Br J Haematol, 1990:186:265.
14. Nagababu E, Rifkind JM. Reaction of hydrogen peroxide with ferrylhemoglobin: superoxide production and heme degradation. Biochemistry,2000:39(40):12503.
15. Wink DA. Mechanisms of the antioxidant effects of nitric oxide. Antioxid Redox Signal,2001:3(2):203-13.
16. Lu N, Chen C, *et al*. The dual effects of nitrite on hemoglobin-dependent redox reactions. Nitric Oxide, 2014:40:1.
17. Hathazi D, Scurtu F, Bischin C, Mot A, *et al*. The reaction of oxyhemoglobin with nitrite: mechanism, antioxidant-modulated effect, and implications for blood substitute evaluation. Molecules,2018:23(2):350.
18. Wilson MT, Reeder BJ. The peroxidatic activities of myoglobin and hemoglobin, their pathological consequences and possible medical interventions. Mol Aspects Med,2022:84:101045.
19. Huseynov TM, Huseynova SY, Guliyeva RT, Dadashov MZ, Rahmanova SM, Jafarova SA. Characteristics of oxidative stress induced by moderate doses of sodium nitrite in isolated erythrocytes in the presence of sodium selenite. Mikroelementy v meditsine,2021:22(2):25−35.