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The reaction of cell-free oxyhemoglobin with nitrite under physiologically relevant conditions: Implications for nitrite-based therapies

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Abstract

Nitric oxide (NO \cdot) participates in the regulation of a wide array of biological processes and its deficit contributes to the severity of many diseases. Recently, a role of NO deficiency that occurs as a result of intravascular hemolysis and increases in levels of cell-free hemoglobin in the pathway of chronic anemic pathologies has been suggested. Experimental evidence for deoxyhemoglobin-catalyzed reduction of nitrite to NO \cdot leads to the possibility of nitrite infusion-based therapies to correct NO \cdot deficits. However, the presence of plasma hemoglobin also raises the possibility of deleterious free radical-mediated oxidative damage from the reaction between nitrite and oxyhemoglobin in the vasculature. We show that the conditions required for the reaction between nitrite and oxyhemoglobin to exhibit free radical-mediated autocatalytic kinetics are highly unlikely to occur in the plasma compartment, even during extensive hemolysis and with pharmacological nitrite doses. Although the presence of haptoglobin enhances the rate of the reaction between nitrite and oxyhemoglobin, common plasma antioxidants—ascorbate and urate, as well as catalase—prevent autocatalysis. Our findings suggest that pharmacological doses of nitrite are unlikely to cause free radical or ferrylhemoglobin formation in plasma originating from the reaction of nitrite with cell-free oxyhemoglobin *in vivo*.

Keywords

Oxyhemoglobin; Nitrite; Ferrylhemoglobin; Nitric oxide; Reactive nitrogen species; Ascorbic acid; Uric acid; Haptoglobin; Cell-free hemoglobin

Introduction

NO \cdot is one of the most potent vasodilators and acts as a signaling molecule in a wide array of pathways in living systems. NO \cdot is classically thought to be generated *in vivo* through the enzymatic activity of the nitric oxide synthases (NOS). NO \cdot itself at physiological conditions is a short lived species. However, its role in signaling processes requires its immediate availability, which implies the existence of storage, or precursor species, with wide availability and lower reactivity, but easy convertibility into active NO \cdot . The most well established precursor is the amino acid L-arginine which is converted to NO through the activity of NOS.

More recently, nitrite ions have emerged as one of the possible precursors of NO \cdot (for review see [1]). Nitrite is present in blood at close to micromolar concentrations (200–500 nM) [2], can be easily and quickly transported by the bloodstream within the body and is much less reactive than NO \cdot . These facts, together with the discovery of nitrite-reductase activities of deoxyhemoglobin (deoxyHb) [1,3–7], nitric oxide synthase [8] and xanthine oxidoreductase [9] suggest that nitrite supplementation may be used to correct NO \cdot deficiency in certain conditions such as stroke or ischemia-reperfusion injury [10–12].

However, significant increase of nitrite levels in plasma could also lead to undesirable oxidative damage within the vascular lumen by free radicals formed in the reaction of plasma oxyhemoglobin with nitrite. The presence of hemoglobin-derived oxidants in plasma had been previously associated with increased endothelial cell damage [13–15]. OxyHb levels in the plasma are in the range of 1 μ M at normal conditions and can reach up to 30 μ M in certain diseases [16]. It was reported that most of the cell-free Hb found in plasma is in ferrous state [17]. Reaction between nitrite and oxyHb may have two potential deleterious effects. First, it may significantly decrease the amount of nitrite available for reduction to NO \cdot by proteins with nitrite-reductase activity, thereby limiting the usefulness of nitrite supplementation. Second, oxidation of oxyHb by nitrite has been shown to be capable of generating an autocatalytic free radical chain reaction that generates ferryl hemoglobin and nitrogen dioxide as intermediates. During the reaction of oxyHb with nitrite, H $_2$ O $_2$ plays a pivotal role in the initiation phase, while the reaction propagation is maintained by the NO $_2$ radical [18]. The end-products are methHb and nitrate. Ferryl species (ferryl-Hb(Fe IV) and the ferrylHb radical) are important intermediates of the process [18–23]. Any extensive uncontrolled formation of free radical species in plasma and their consequent reactions with various biomolecules could result in tissue and organ damage [13–15]. However, most studies that have examined the kinetics and mechanism of this reaction have used a large excess of nitrite over Hb (10– to 100-fold), or oxyHb concentrations in the millimolar range. As the reaction mechanism is complex, the concentration dependencies examined at non-physiological reactant concentrations and ratios can not be extrapolated to the concentrations and ranges found in the physiological or pharmacological arena. It is therefore important to study this reaction under conditions expected to be found in the plasma in hemolytic disorders.

In case of hemolysis, cell-free hemoglobin and heme are rapidly cleared from bloodstream by highly specialized proteins, haptoglobin (Hp) and hemopexin, respectively [24,25]. Hp is a major Hb-binding protein in plasma, so we further focused on the effect of this protein. In this study, we used dimeric Hp 1-1 with two binding sites for Hb dimers, which is present in human blood at concentration \sim 20 μ M. Hp 1-1 has a high affinity for Hb ($K_d \sim 10^{12}$ M), and the resulting complex is very stable [24,26]. The physiological role of Hp–Hb complex formation seems to be mainly as aid in iron recycling from Hb by circulating leukocytes, and in preventing renal iron loading from Hb [27]. It has been reported that the Hb–Hp complex retains NO \cdot -scavenging activity [17,28,29] and its peroxidase activity is higher than the activity reported for Hb alone [30,31]. It is believed that Hp–Hb complex formation prevents oxidative damages caused by free Hb in vasculature [32,33].

In this paper, we used EPR to detect free radical species in plasma with added oxyHb and nitrite at equimolar Hb/nitrite ratios, and compared it to the reaction at a 10-fold excess of nitrite. In neither case were free radical species detected. When the same reaction was carried out in phosphate buffer, radical species formation was observed at the 10-fold excess of nitrite. Interestingly, methHb formed faster in plasma than in buffer at equimolar nitrite/oxyHb ratio and haptoglobin enhanced the reaction rate even at low nitrite regime. Systematic study of this reaction at a wide array of nitrite/oxyHb molar ratios, and the examination of the effect of various active compounds of plasma, such as antioxidants, haptoglobin and catalase, allowed us to determine conditions where the process exhibits full autocatalytic character and to clarify

the importance of these species for free radical formation and suppression. A detailed understanding of possible redox reactions of cell-free hemoglobin in plasma upon nitrite addition is necessary for successful design of safe nitrite-based therapies for NO \cdot deficiency.

Materials and methods

Materials

Fresh blood drawn from a healthy volunteer into test tubes containing heparin was immediately centrifuged for 20 min at 3000g to separate plasma from other blood components. Separated plasma was used immediately for EPR experiments.

OxyHb was prepared from fresh human blood according to Rossi-Fanelli et al. [34]. Hb aliquots were frozen in liquid nitrogen and stored at -80°C . Sodium nitrite, ascorbic acid, uric acid, human haptoglobin phenotype 1-1 and catalase from bovine liver were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Phosphate buffered saline (PBS, 10 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4) contained 1 mM DTPA.

Electron paramagnetic resonance (EPR) spectroscopy

To determine if free radical formation from studied reaction occurs at physiological conditions, EPR experiments were carried out in plasma and PBS. For experiments done in plasma, oxyHb from stock solution (3 mM, PBS) was added into freshly separated plasma (final concentration of 30 μM). Samples with nitrite/oxyHb molar ratios of 1/1 and 10/1 were prepared in triplicates adding appropriate amounts of stock solution of NaNO $_2$ (100 mM, in PBS). Plasma with added oxyHb and nitrite was incubated at 37 $^{\circ}\text{C}$ and aliquots for EPR experiments were withdrawn at the following time points: for nitrite/oxyHb equimolar ratio: 0, 1, 4, 6 and 11 h, and for nitrite/oxyHb molar ratio of 10/1 at 0, 15, 30, 45, 60, 90, 120 and 300 min after the beginning of incubation. The aliquots were immediately frozen in liquid nitrogen and stored at -80°C until the analysis. Both, methHb and free radicals were measured.

For experiments done in PBS, samples with nitrite/oxyHb molar ratios of 2/1 and 10/1 were prepared and measured. Aliquots were taken at following time points: 2, 12 and 24 h for nitrite/oxyHb molar ratio of 2/1, and at 2, 3 and 6 h for nitrite/oxyHb molar ratio of 10/1.

Methemoglobin (methHb) was detected in $g = 6$ region at the temperature of 6 K using a Bruker Elexys EPR spectrometer (Bruker, MA), at microwave power of 2 mW, modulation amplitude 10 G, sweep time 84 s. Same samples were then scanned in $g = 2$ region at temperature of 90 K to detect free radical species using Bruker EMX EPR spectrometer (Bruker, MA) equipped with a loop-gap resonator, at microwave power 0.2 mW, modulation amplitude 2 G and sweep time 84 s.

The amount of methHb in samples in EPR measurements was calculated using freshly prepared methHb standard.

UV-visible spectroscopy

To follow and identify reaction products in reaction of oxyHb with nitrite in PBS at 37 $^{\circ}\text{C}$, the concentration of oxyHb was kept constant at 30 μM . The appropriate amounts of stock solution of NaNO $_2$ (100 mM, in PBS) were injected into oxyHb solution to obtain desired final nitrite concentrations. UV/vis spectra were automatically acquired in 450–700 nm range at every 15–360 s, depending on studied nitrite/Hb ratio. The following nitrite concentrations were used: 0, 3, 6, 15, 30, 60, 120, 300 and 450 μM , corresponding to nitrite/oxyHb molar ratios of 0,

1/10, 1/5, 1/2, 1/1, 2/1, 4/1, 10/1 and 15/1, respectively, and most reactions were carried out until completion. At conditions with completion times of more than 24 h at least one experiment was carried out to completion, otherwise experiments were stopped at 18–24 h marks for technical reasons. All spectra were recorded using UV/vis diode array spectrophotometer model HP 8453 (Agilent Technologies, Santa Clara, CA) equipped with 8-cell transporter and water bath temperature control. Samples were prepared and measured in triplicate for each nitrite/oxyHb ratio.

To examine the effect of ascorbic acid (AA) and uric acid (UA), we added freshly prepared solution of AA/UA into samples with 30 μM oxyHb to final concentrations of 5, 50, 100 and 500 μM for AA and 50, 100, 250 and 500 μM for UA. Immediately after the AA (UA) addition, nitrite was added to the final nitrite/oxyHb ratio of 10/1 and absorption spectra were acquired for 18 h with time intervals of 4 min.

To examine the role of H_2O_2 in radical chain formation, catalase from bovine liver was added at the beginning of experiment in appropriate amounts to achieve final activity of 50 kU/ml.

Human haptoglobin 1-1 (Hp) was added to a final concentration of 20 μM into solution of 14 μM or 7 μM oxyHb. Samples with nitrite/oxyHb molar ratios of 2/1 and 10/1 were prepared and kinetics of the reaction was followed using absorption spectroscopy. Control sample containing Hp/Hb without nitrite and samples of 14 μM or 7 μM Hb with added nitrite was included in each experimental set.

Multiple linear regression analysis (MLR) approach was used to determine contributions of each Hb species into the measured UV–vis spectra at each time point. A reference set of pure oxyHb, methHb and ferrylHb spectra were used (Fig. 1B) and the quality of fit was estimated using the plot of residuals. An illustrative spectral data set for nitrite/oxyHb molar ratio of 2/1 is shown in Fig. 1A, where arrows indicate temporal evolution of individual peaks and gray lines are plots of residuals after the MLR fit.

Results

Absorption spectra were recorded during the reaction of oxyHb with nitrite in PBS, in a series of experiments with variable nitrite/oxyHb ratios at a constant oxyHb concentration of 30 μM . Contributions of individual Hb species were determined using MLR analysis using the basis spectra shown in Fig. 1. A complete plot of changes in the fraction of oxyHb as a function of time, for different nitrite/Hb molar ratio in PBS is in Fig. 2A. In the absence of nitrite slow autoxidation of oxyHb was observed. As expected, the reaction proceeds faster as nitrite concentrations increases, however, the kinetic profile was also dependent on nitrite concentration. At nitrite/oxyHb ratios below 4:1, oxyHb decay was monophasic. At ratios of 4:1 and greater, the reaction kinetics exhibited the autocatalytic profile that has been previously observed. Initial rate plotted as a function of nitrite concentration gave a bimolecular rate constant of $0.21 \text{ M}^{-1} \text{ s}^{-1}$ for the rate limiting step of the initial reaction (Fig. 2B).

Full deconvolutions of the spectral changes observed during the reaction between nitrite and oxyHb are shown in Fig. 3. Each species is given in terms of its fractional contribution to the total amount of hemoglobin present. Under these experimental conditions oxyHb is oxidized only by a slow monotonic process, and the ferrylHb intermediate never achieves measurable levels. The removal of the ferryl species from the basis spectra did not alter the quality of fit (data not shown). EPR spectra in the $g = 2$ region did not reveal the formation of protein free radicals under these conditions (Fig. 3B). In the case of 10-fold excess of nitrite over oxyHb (Fig. 3C), all characteristics of an autocatalytic reaction were observed. The spectral deconvolution revealed a transient formation of ferryl hemoglobin species during the rapid

phase of the reaction. In addition, a transient protein-based free radical coinciding with the fast phase of reaction was detected in $g = 2$ region (Fig. 3D).

MetHb formation in plasma, detected by EPR spectroscopy, during the reaction of $30 \mu\text{M}$ oxyHb with nitrite at an equimolar nitrite/oxyHb ratio is shown in Fig. 4. EPR spectra for plasma samples in the metHb ($g = 6$) and free radical ($g = 2$) regions are shown in Fig. 4A and B, respectively. The development of the metHb signal with increased time of incubation is clearly seen in Fig. 4A as a function of time, however, no EPR signals were observed in the free radical region. In an attempt to assess nitrite concentration dependence of the reaction kinetics in plasma, a sample with an excess of nitrite at nitrite/oxyHb molar ratio of 10/1 was prepared and measured by EPR in $g = 6$ and $g = 2$ regions. The transient presence of metHb EPR signal during the course of the reaction was detected in the $g = 6$ region and no free radicals were detected in the $g = 2$ region (data not shown). The concentration of metHb in plasma was calculated using a metHb standard and is plotted in Fig. 4C as a function of the incubation time. For comparison, metHb concentration changes occurring during the reaction of oxyHb with nitrite in PBS under otherwise identical experimental conditions are also shown. Interestingly, the presence of plasma increased the rate of metHb formation by almost 2-fold.

Fig. 5 summarizes the effect of presence of $20 \mu\text{M}$ haptoglobin (Hp) on the decay of oxyHb during its reaction with nitrite at equimolar nitrite/oxyHb ratio at an oxyHb concentration of $14 \mu\text{M}$. The presence of Hp does not alter autooxidation of oxyHb in the absence of nitrite (lines a vs. b). In the absence of Hp, nitrite causes only monotonous oxyHb decay (line g). Interestingly, the presence of Hp in the reaction mixture of oxyHb resulted in biphasic kinetics (line h). Addition of $50 \mu\text{M}$ ascorbic acid significantly slows oxyHb decay in presence as well in the absence of Hp (lines c and d). A similar effect was found for $100 \mu\text{M}$ uric acid (data not shown). Addition of catalase (50 kU/ml) at the beginning of the experiment decreases the reaction rate regardless of Hp presence/absence (line e and f). Similar effects were observed during the reaction between $7 \mu\text{M}$ Hb, $7 \mu\text{M}$ nitrite, and $20 \mu\text{M}$ Hp (not shown). Addition of Hp into nitrite/oxyHb reaction mixture with 10-fold nitrite excess (a condition in which the reaction exhibits autocatalytic kinetics), causes dramatic shortening of the slow phase and acceleration of the development of rapid phase (data not shown).

Fig. 6A and B show the potency of ascorbic and uric acids, respectively, to prevent the development of the rapid phase in reaction of oxyHb with nitrite at 10-fold excess of nitrite. Both antioxidants were tested at wide range of concentrations. As plotted in Fig. 6A, the presence of $5 \mu\text{M}$ ascorbic acid was enough to elongate the duration of slow “lag” phase from 3 h (without ascorbic acid) to 15 h before the reaction finally entered into the rapid phase. Further increases of ascorbic acid concentration to 50, 100 and $500 \mu\text{M}$ completely prevented the appearance of the rapid phase. Fig. 6B depicts the similar results observed when uric acid was used. All tested concentrations, 50, 100, 250 and $500 \mu\text{M}$ effectively prevented the development of the rapid phase of reaction.

Discussion

The reaction of nitrite with oxyHb can involve multiple steps with complex concentration dependencies and many intermediate products. The reaction kinetics then are bimodal, with a slow phase (initiation), during which oxyHb is oxidized to metHb, that precedes a rapid propagation phase. The process is autocatalytic, as the propagation species is generated in multiple copies during one reaction cycle. Earlier attempts to explain the mechanism have been reviewed [35]. Our recent kinetic reexamination of this reaction suggests that the most crucial intermediates, H_2O_2 and NO_2 radical act exclusively in the initiation and the propagation phases, respectively [18]. In this study we have attempted to examine this reaction in the context of nitrite supplementation in hemolytic disorders, and to ascertain if the oxidative intermediates

generated during the progression of this reaction are likely to be of concern. For this reason we have focused on relevant concentration ranges of hemoglobin (up to 30 μM in plasma [16]) and on plasma components that may alter the reaction characteristics. As shown in this study, common plasma antioxidants (AA, UA) regulate the reaction by limiting the quantity of intermediate reactive products (as H_2O_2 , NO_2 radical, ferrylHb species).

Our EPR and spectrophotometric results for equimolar nitrite/oxyHb mixtures indicate that no free radicals are detected during the whole time course of the reaction. When 10-fold excess of nitrite over oxyHb was used, the situation in plasma and PBS *vis a vis* free radical formation differed dramatically; with an total absence of free radicals in plasma and clear transient presence of protein-based radical in PBS that coincided with the observed fast phase of the reaction (Fig. 3D). The observed monotonous kinetics of the reaction up to ~4-fold nitrite excess over oxyHb suggests that a burst of oxidizing reactive intermediates does not occur.

Natural levels of nitrite in plasma are in the nanomolar range of 200–500 nM [2]. Nitrite/oxyHb molar ratios of 1/1 and 10/1 in our study were chosen with two possible nitrite supplementation routes in mind. Supplementing nitrite by dietary sources with green leaf vegetables as a preferred source, 100 g of spinach would lead to total nitrite dose in range of 1 $\mu\text{mol/kg}$ [1], or around 1 μM nitrite in blood. Therefore, concentrations of nitrite in plasma would unlikely to exceed its natural levels much more than 5-fold by dietary changes only. In this case, with extracellular oxyHb in plasma of ~1.4 μM , diet-modified nitrite/oxyHb molar ratio is ~1/1 without hemolysis and drops to ~1/30 during the hemolysis, so free radical formation in plasma is not expected. However, in case of acute NO^- deficiency, more direct route to deliver nitrite is needed. In nitrite infusion-based therapies much higher levels of nitrite in plasma are possible, which could potentially lead to the possibility of free radical formation in the vasculature, such as ferrylHb species [13–15]. However, vasodilatory effects of nitrite occur even under a low pharmacologic concentration regime which is unlikely to generate a burst of oxidizing species. Infusion of nitrite into human forearm brachial artery to its final concentration in blood of 2 μM caused 30% increase of blood flow [4]. When 100-fold higher concentration of nitrite was used (200 μM), much higher increase of blood flow was observed (175%), but one could argue that achieving such a large increase in blood flow could be unnecessary or even dangerous in some cases. Also, intravenous infusion instead of intra-arterial could take advantage of the higher deoxyHb content of venous blood to make it possible to lower the necessary dose.

Reactive free radical species can be scavenged by antioxidants present in plasma. The two main antioxidants in plasma are ascorbic acid and uric acid with physiological concentration range of 20–60 μM and 300–400 μM , respectively [36–38]. The ability of both to destroy or at least significantly limit free radicals formed in case of 10-fold excess of nitrite over oxyHb in PBS suggests that the risk of free radical formation in nitrite-based therapies can be lowered by concurrent administration of antioxidants as previously shown [39]. FerrylHb and free radicals which are potential cause of oxidative stress in the vasculature, seem to be formed only at very non-physiological conditions of nitrite, such as may occur during nitrite poisoning [40] and should not play a major role in most processes involving cell-free plasma Hb *in vivo*.

Interestingly, at conditions of equimolar nitrite and oxyHb, the rate of metHb formation was significantly higher than in PBS (Fig. 4C). At normal conditions, cell-free hemoglobin and heme are rapidly bound to haptoglobin (Hp) and hemopexin, respectively [24,25] and cleared out of the blood. We hypothesized that the increased rate of metHb formation observed in plasma might be caused by the presence of Hp. Earlier it had been reported that heme environment in Hb in Hp–Hb complex is slightly altered when compared to free Hb [41,42]. Also, Hp binds primary to the Hb dimer.

It is known that the Hb–Hp complex retains NO· scavenging activity [17,28,29], but to our best knowledge no report had been made on how Hb-binding to Hp affects Hb reaction with nitrite ion. Several studies also report that Hp–Hb complex formation prevents oxidative damages caused by free Hb in vasculature [32,33].

Based on observed accelerated metHb formation from cell-free Hb in plasma (Fig. 4C) we further examine the effect of Hp presence on Hb-nitrite reaction kinetics. When the Hp–Hb complex was incubated with nitrite, we observed biphasic decay with a ferrylHb intermediate clearly present in MLR deconvolution (Fig. 5). This observation is in agreement with published data [43], where formation of ferrylHb from oxyHb as well as from metHb was not inhibited, but increased by Hp–Hb complex formation. However, according to the proposed reaction mechanism for oxyHb with nitrite [18], as well as the observed acceleration of metHb formation in plasma, we would expect to see some traces of protein-based free radicals in plasma, at least in the case of 10-fold nitrite excess, which we did not observe. The main difference between PBS and plasma in this regard is likely to be the presence of plasma antioxidants. To examine this hypothesis we used antioxidants added to the reaction mixture of nitrite/Hp–Hb in PBS at the beginning of the reaction. Indeed, after addition of 50 μ M ascorbic acid and 100 μ M uric acid only slow monotonic oxyHb decay, but not autocatalysis was observed.

Catalase added at the beginning of the reaction also prevented the autocatalytic burst both in presence and in absence of Hp, showing that Hp did not open an alternative reaction path leading to autocatalysis. We assume that formation of Hp–Hb complex with Hb dimer conformation in R state is responsible for enhancing the reaction kinetics.

In this study we provide insight into reaction of cell-free Hb with nitrite at low oxyHb and nitrite regimes as expected in plasma at physiological and pharmacological ranges and we also examine possible interactions with other plasma components. We found no autocatalytic burst of oxidant formation under these conditions, unlike what has been observed at high nitrite:Hb ratios and/or high Hb concentrations [19,44–48]. The presence of ferrylHb-based radicals was confirmed in case of 4-fold and higher excess of nitrite over oxyHb, and at low nitrite:oxyHb ratios when Hp–Hb complex was formed prior to the reaction. Addition of ascorbic and uric acids at concentrations normally found in plasma abolished radical formation, and added catalase scavenged H₂O₂. We conclude that effective pharmacological doses of nitrite, especially when complemented with antioxidant effects of plasma, are not likely to be limited *vis a vis* free radical or ferrylHb formation by this reaction *in vivo* during nitrite-based therapies in diseases accompanied by hemolysis or otherwise due to NO· deficiency [49,50]. Our study concentrated on cell-free hemoglobin, however, the major part of hemoglobin found in blood is of course encapsulated in red blood cells. One can ask about the consequences of nitrite infusion on this portion of hemoglobin. Based on the overwhelming ratio of hemoglobin over nitrite and the presence of antioxidants and metHb reductase system inside the cell we would predict that therapeutically desirable increases of nitrite concentration in plasma would have only a small effect on redox processes inside the red blood cell. Based on the results from our *in vitro* study, we believe that for the case of nitrite infusion therapy used for diseases accompanied by hemolysis, the determining factor *vis a vis* radical formation in the vasculature would be the cell-free hemoglobin. The risk of free radical formation in blood in direct nitrite infusion-based therapies can be further lowered by co-administration of sufficient doses of naturally occurring antioxidants, such as ascorbic acid (which itself could potentially participate in the reduction of nitrite to NO). However, this study concerns itself only with the reactions of nitrite in the plasma and therefore can not completely rule out any potentially detrimental effects of nitrite that may result from alternative routes of administration. Our results also suggest that pharmacological doses should be relatively low, below the nitrite/free Hb molar ratio of 4; however, to give recommendations for doses, a clinical trial is necessary.

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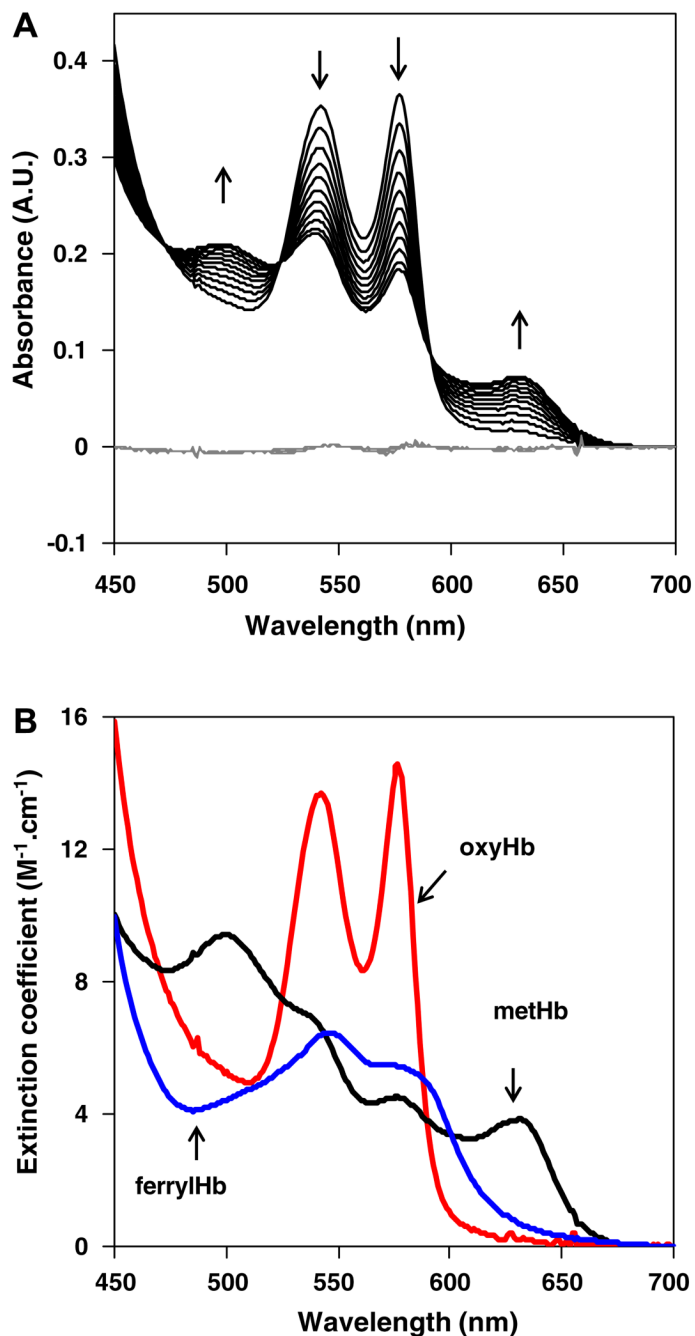


Fig. 1.

(A) Reaction of oxyHb with nitrite at nitrite/oxyHb molar ratio of 2/1 followed continuously for 20 h. OxyHb concentration $30\ \mu M$, nitrite $60\ \mu M$, PBS, $37\ ^\circ C$, pH 7.4, absorption spectra were recorded every 5 min, for clarity only spectra taken in 2 h intervals are plot. Directions of changes in absorption spectra of hemoglobin (black lines) in time are marked by arrows. The residuals between MLR analysis using set of absorption spectra from (B) are plot as gray lines. (B) Standard absorption spectra of pure oxyHb, metHb and ferrylHb used in MLR analysis.

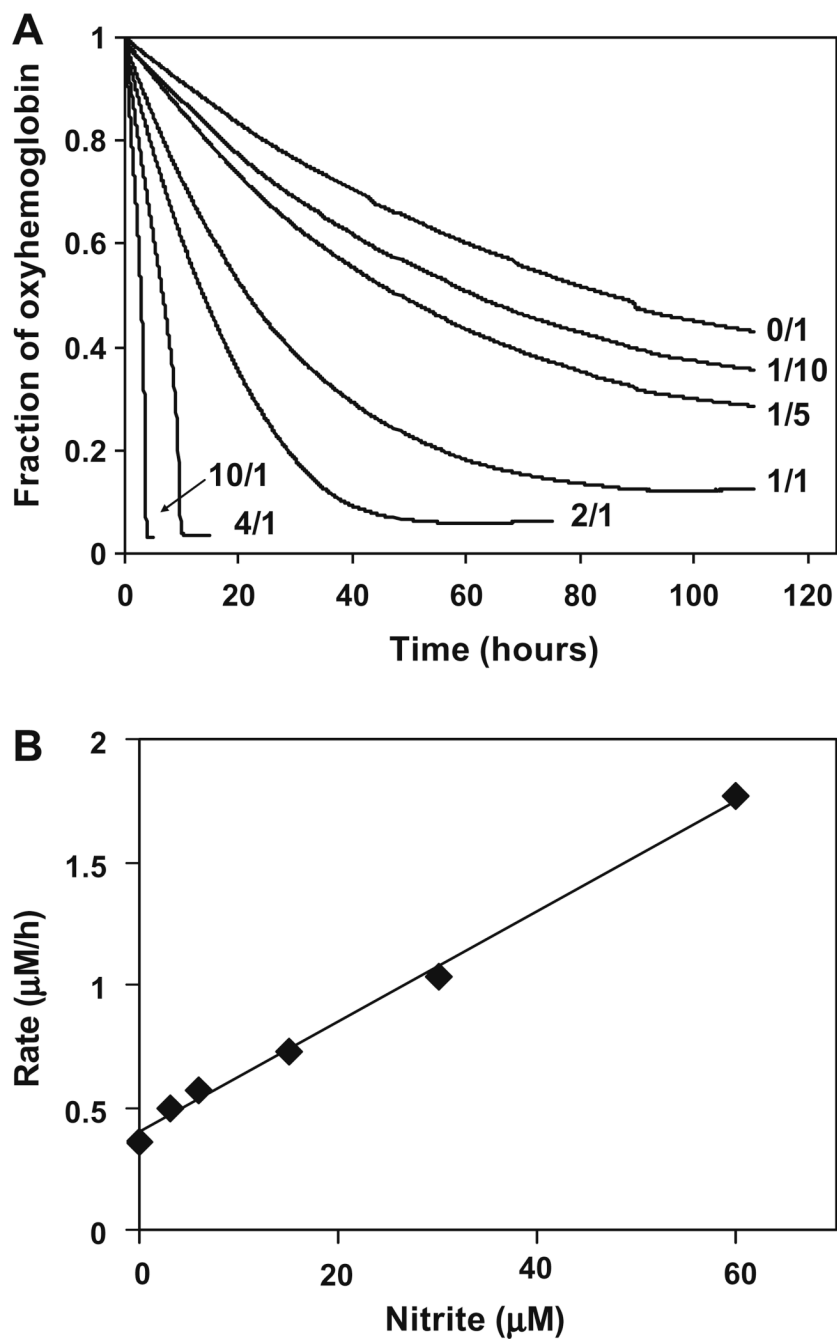


Fig. 2. (A) OxyHb decay as function of time after addition of nitrite ions at different nitrite/oxyHb molar ratios in PBS at 37 °C and pH 7.4 and oxyHb concentration 30 μM . Two phase oxyHb decay was observed for nitrite/oxyHb molar ratios $\geq 4/1$. Number on the right denotes the corresponding nitrite/oxyHb molar ratio. (B) Initial rates of oxyHb decay for all nitrite/Hb molar ratios studied. Hb concentration was kept constant at 30 μM in and all experiments were done in PBS at 37 °C and pH 7.4.

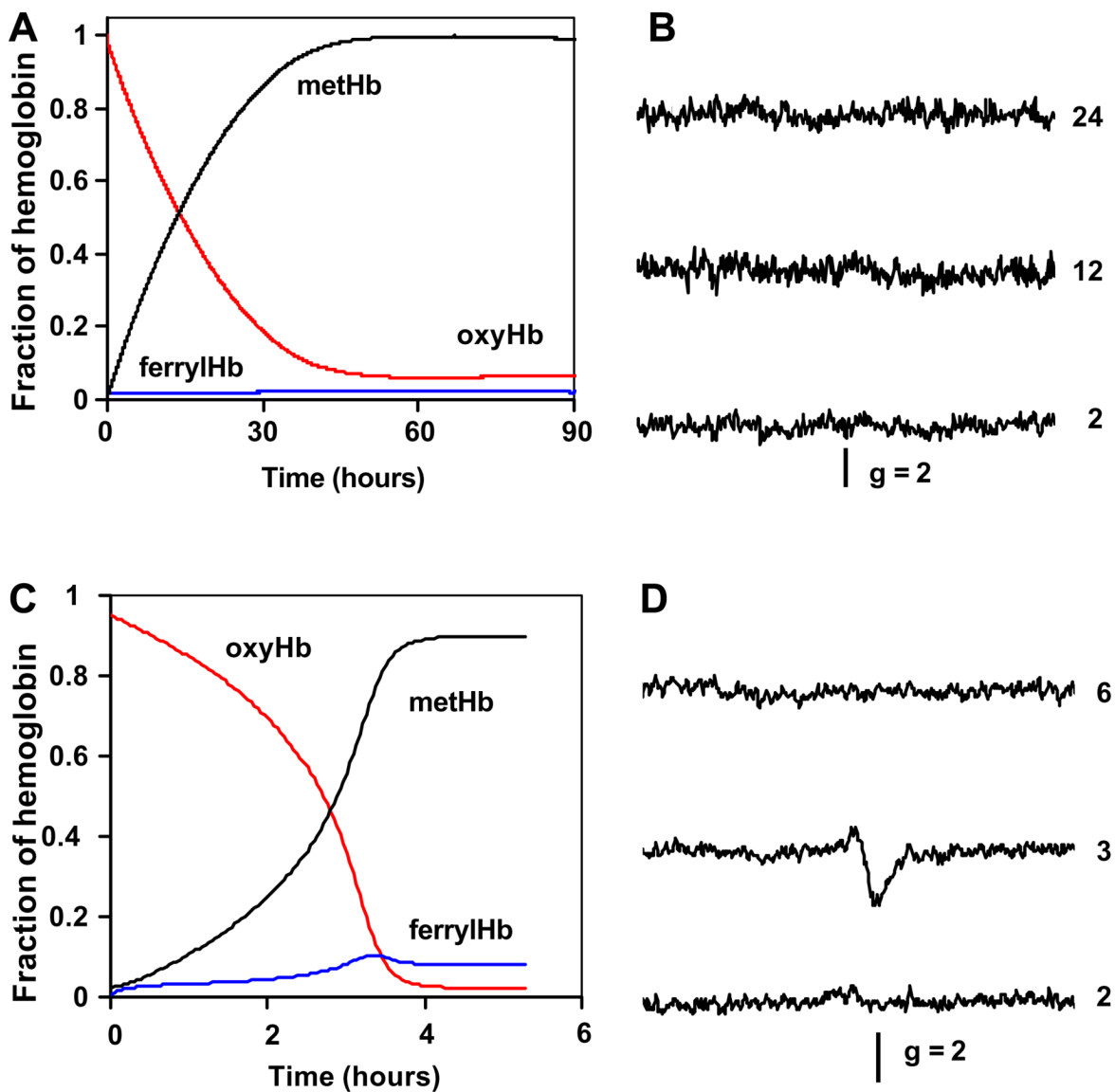


Fig. 3. (A) Multilinear regression analysis applied to the spectral set from Fig. 1A using standard oxyHb, ferrylHb and methHb spectra. (B) EPR scans in $g = 2$ region of aliquots taken from reaction mixture of nitrite/oxyHb 2/1 at different time points indicated by numbers on the right (time given in hours) (C) Multilinear regression analysis applied to the spectral set from experiment with nitrite/Hb molar ratio of 10/1 (300 μ M nitrite and 30 μ M oxyHb) using standard oxyHb, ferrylHb and methHb spectra. (D) EPR scans in $g = 2$ region of aliquots taken from reaction mixture of nitrite/oxyHb 10/1 at different time points indicated by numbers on the right (time given in hours).

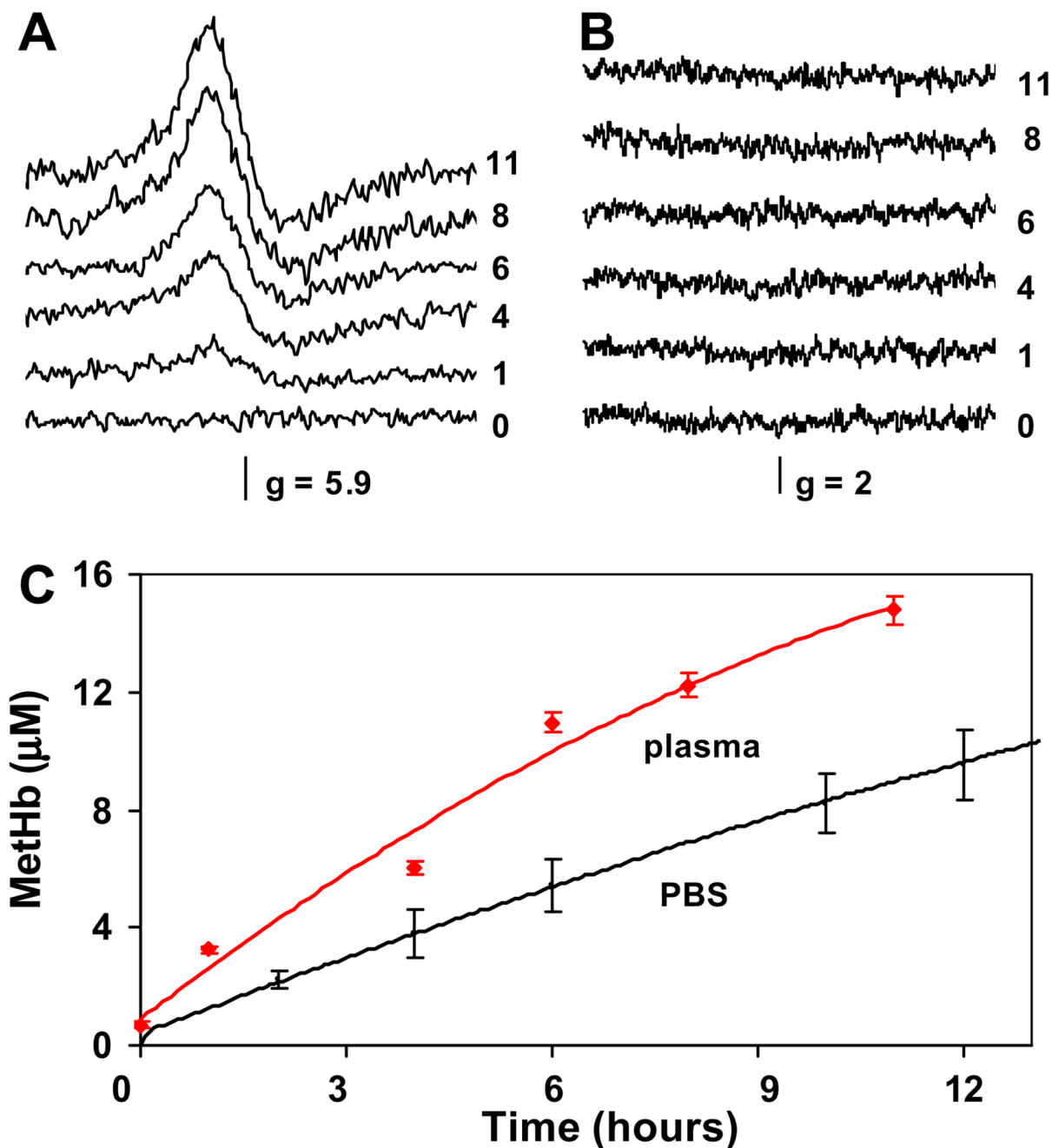


Fig. 4. (A) MetHb EPR spectra of aliquots taken from the reaction mixture of equimolar ratio of nitrite/oxyHb 1/1 in plasma at different time points indicated by numbers on the right (time given in hours). (B): EPR spectra of the same aliquots as in (B) scanned for the presence of free radical species in $g = 2$ region. (C) MetHb formation in plasma with added cell-free oxyhemoglobin after nitrite addition as determined using EPR spectroscopy (red line) compared with metHb formation in the same reaction carried on in PBS (pH 7.4) and followed using absorption spectroscopy (black line). Reaction conditions in both cases: oxyHb $30 \mu\text{M}$, nitrite $30 \mu\text{M}$, 37°C . (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

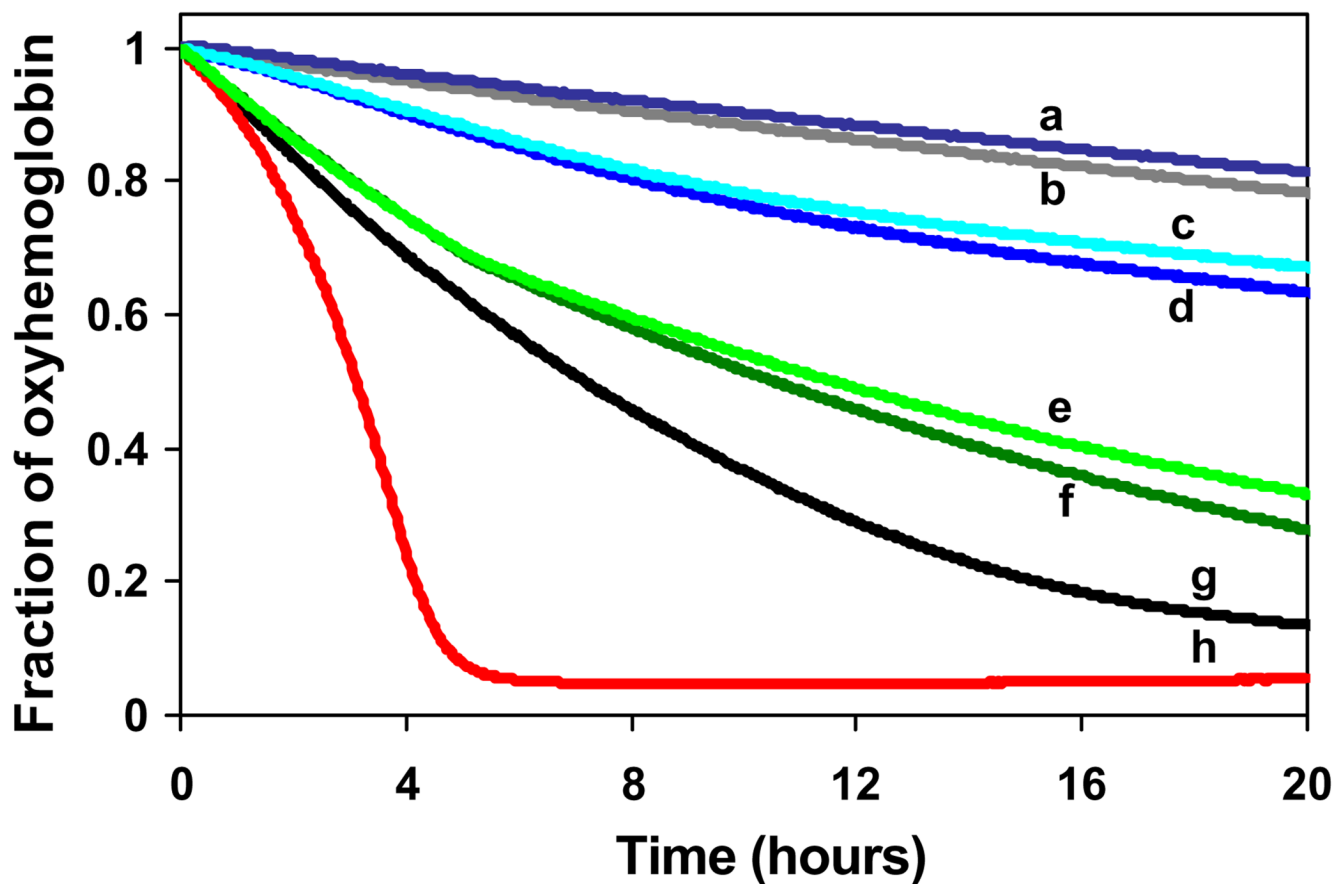


Fig. 5. Comparison of effects of ascorbic acid (AA) and catalase on the kinetics of oxyHb reaction with nitrite at equimolar ratio in presence/absence of Hp in PBS 37 °C. (a) oxyHb/Hp; (b) oxyHb; (c) oxyHb/Hp/nitrite/AA; (d) oxyHb/nitrite/AA, (e) oxyHb/nitrite/catalase; (f) oxyHb/ Hp/nitrite/catalase; (g) oxyHb/nitrite; (h) oxyHb/Hp/nitrite. Following concentrations of reactants were used: oxyHb 14 μ M, nitrite 14 μ M, Hp 20 μ M, AA 50 μ M and catalase 50 kU/ml.

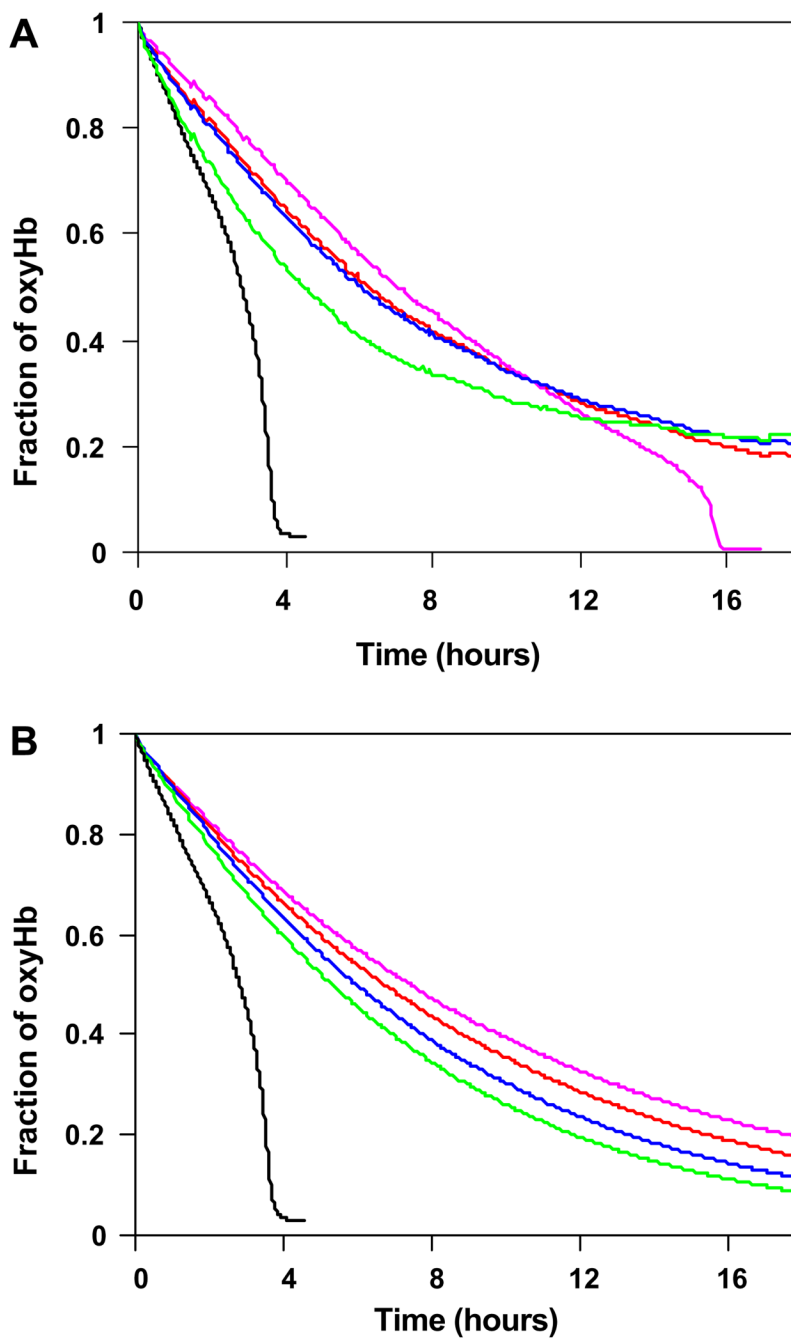


Fig. 6. (A) Effect of ascorbic acid on the reaction of nitrite with oxyHb. Experimental conditions: ascorbic acid 0 μM (black), 5 μM (purple), 50 μM (blue), 100 μM (red) and 500 μM (green), oxyHb 30 μM , nitrite/oxyHb 10/1, PBS, pH 7.4, 37 $^{\circ}\text{C}$. (B) Effect of uric acid on the reaction of nitrite with oxyHb. Experimental conditions: uric acid 0 μM (black), 50 μM (purple), 100 μM (red), 250 μM (blue), 500 μM (green), oxyHb 30 μM , nitrite/oxyHb 10/1, PBS, pH 7.4, 37 $^{\circ}\text{C}$. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)