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Clin Hemorheol Microcirc. Author manuscript; available in PMC 2019 January 01.

Published in final edited form as:
Clin Hemorheol Microcirc. 2018 ; 68(2-3): 223–237. doi:10.3233/CH-189009.

Nitric Oxide Pathology and Therapeutics in Sickle Cell Disease

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Abstract

Sickle cell disease is caused by a mutant form of hemoglobin that polymerizes under hypoxic conditions which leads to red blood cell (RBC) distortion, calcium-influx mediated RBC dehydration, increased RBC adhesivity, reduced RBC deformability, increased RBC fragility, and hemolysis. These impairments in RBC structure and function result in multifaceted downstream pathology including inflammation, endothelial cell activation, platelet and leukocyte activation and adhesion, and thrombosis, all of which contribute vascular occlusion and substantial morbidity and mortality. Hemoglobin released upon RBC hemolysis scavenges nitric oxide (NO) and generates reactive oxygen species (ROS) and thereby decreases bioavailability of this important signaling molecule. As the endothelium-derived relaxing factor, NO acts as a vasodilator and also decreases platelet, leukocyte, and endothelial cell activation. Thus, low NO bioavailability contributes to pathology in sickle cell disease and its restoration could serve as an effective treatment. Despite its promise, clinical trials based on restoring NO bioavailability have so far been mainly disappointing. However, particular “NO donating” agents such as nitrite, which unlike some other NO donors can improve sickle RBC properties, may yet prove effective.

Keywords

Nitric oxide; Sickle cell disease; hemolysis; red blood cell; hemoglobin

1. Sickle Cell Disease

Sickle cell disease (SCD) is the most common genetic disease affecting about 2600 births a year in North America [1]. It is caused by a single mutation in the gene coding for the beta subunit of hemoglobin (Hb) resulting in substitution of hydrophobic valine for hydrophilic glutamate at the 6th position [2]. This mutant form of Hb, known as sickle cell Hb (HbS) polymerizes when the Hb is in the low oxygen affinity T-state [3]. The HbS polymers increase the intracellular viscosity of the red blood cells (RBCs) and distort the shape of the

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RBCs, causing membrane damage, decreased RBC deformability and increased fragility [4]. The distortion of the shape of cells caused by polymerization is referred to as sickling.

HbS polymers form upon deoxygenation since T-state HbS is the quaternary structure that polymerizes so that sickling occurs as the RBCs enter areas of hypoxia [5–7]. When RBCs are re-oxygenated at the lungs, and Hb binds oxygen forming R-state Hb, the polymers can melt [8, 9]. The amount of HbS that polymerizes when equilibrium is reached is defined by the solubility C_s and the total Hb concentration C_0. In vivo, the primary factor effecting the solubility is Hb oxygen saturation, while the percentage of non-polymerizing hemoglobins (such as fetal Hb) also increases the solubility dramatically [3]. The HbS exceeding the solubility goes into the polymer phase. HbS polymerizes via a double nucleation mechanism whereby once a critical nucleus of HbS molecules is formed, addition of another Hb molecule is thermodynamically more favorable than dissociation of Hb from the aggregate, so that polymerization is favored [10, 11]. Nucleation then also occurs on the growing polymer so that the kinetics of polymerization is characterized by little to no polymers for a period of time known as the delay time, t_d, followed by rapid exponential growth [3]. The delay time is related to the solubility as

$$\frac{1}{t_d} = \lambda\left(\frac{c_0}{c_s}\right)^n, \quad (1)$$

where λ is a constant of proportionality and the exponent n ranges from 15 to over 30 [3, 12, 13]. The delay time is thus extremely sensitive to the solubility and total mean corpuscular hemoglobin concentration (MCHC) so that, according to the kinetic theory, a small decrease in C_0 / C_s (either by decreasing MCHC or increasing the solubility) can dramatically increase the delay time so that RBCs can successfully traverse the vasculature and return to the lungs without initiation of polymerization. It should be mentioned that the importance of the delay time in pathology of SCD has been debated where it has been suggested that the equilibrium value of C_0 / C_s is the most relevant indicator of pathology based on findings that many sickle RBCs contain polymers even on the arteriolar side of the microcirculation [14, 15].

Cycles of sickling and unsickling result in accumulating membrane damage that leads to calcium influx [16, 17]. As further described in another article in this special issue of Clin Hemorheol Microcirc [see the review of Brugnara] calcium activates the Gardos channel resulting in potassium export and dehydration of the RBC [16, 17]. Cellular dehydration decreases RBC deformability and increases MCHC. Increasing MCHC promotes polymerization as C_0 increases [10, 11, 18]. Thus, dehydration is part of several vicious cycles in the pathology of SCD, where increased dehydration leads to more polymerization which leads to more membrane damage, calcium influx, and back to more dehydration. Another consequence of sickling and dehydration induced membrane damage is the loss of RBC membrane phospholipid asymmetry that is associated with increased adhesivity of sickle RBCs [19, 20].

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Thus, HbS polymerization is the primary pathological event in SCD which results in RBC rigidification and increased fragility. Increased RBC fragility leads to hemolysis which contributes to oxidative stress and platelet activation [21, 22]. The oxidative stress, physical interactions of sickled cells with the endothelium and other factors result in an inflammatory state where endothelial cells and leukocytes are activated resulting in leukocyte and multi-cellular adhesion to the endothelium [23–26]. These multi-cellular aggregates cause vascular occlusion that results in ischemia, organ damage and death [26]. For further details on these mechanisms, see the reviews of Van Beers and Van Wijk, Faes and colleagues, and Brittain et al in this issue of Clin Hemorheol Microcirc.

2. Nitric Oxide

Nitric oxide (NO) is a free radical, diatomic, gas molecule. It is very soluble in water (about 2 mM at room temperature) and about 7-fold more soluble in non-polar solvents and biological lipid membranes, so that it can easily traverse phospholipid membranes. In fact the diffusion constant is quite high, as expected for a small molecule; 3300 $\mu m^2/s$ [27], yet (as discussed below) some reactions involving NO are diffusion-rate limited. The wide range of biological functions that NO contributes to are partially due to its ability to undergo redox reactions and form several congeners illustrated in Table 1.

NO is produced from arginine by nitric oxide synthases [28],

$$2\ \text{arginine} + 3\ \text{NADPH} + 1H^+ + 4O_2 \rightarrow 2\ \text{citrulline} + 2\ \text{NO} + 4H_2O + 3\ \text{NADP}^+ \quad (2)$$

where NADP is nicotinamide adenine dinucleotide phosphate. There are three types of NOSs: neuronal NO synthase (NOS1), macrophage inducible NOS (NOS2), and endothelial NOS (NOS3) [29, 30]. Substantial evidence suggests that there is also a functional endothelial NOS inside the RBC [31–33]. NO can also be formed by redox reactions involving one of its congeners (Table 1). The most important one of these reactions biologically is the reduction of nitrite to NO. Several enzymes have been proposed to be involved in nitrite reduction including hemoglobin [34, 35], myoglobin [36, 37], xanthine oxidoreductase [38, 39], nitric oxide synthase [40], carbonic anhydrase [41], cytochrome c oxidase [42, 43], cyclooxygenase [44], aldehyde oxidase [45, 46] and cytochrome c [47, 48]. The reaction of hemoglobin with nitrite may be particularly relevant to SCD where a ligand-free, ferrous heme (as in deoxygenated Hb, (deoxyHb)) accepts an electron from nitrite [49–51],

$$NO_2^- + HbFe^{2+} + H^+ \rightarrow NO + HbFe^{3+} + OH^-, \quad (3)$$

where $HbFe^{2+}$ refers to a ferrous, unlganded heme of Hb and $HbFe^{3+}$ refers to a ferric heme that is characteristic of methemoglobin. Thus, whereas NOS requires oxygen to produce NO, nitrite produces NO when oxygen tension is lowered so that Hb becomes partially deoxgenated.

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Nitrite is produced by NO's reaction with oxygen and other mechanisms [52, 53]. It is normally found at a blood concentration of up to about 0.5 μM [54]. It has been historically viewed as an inert byproduct [55], but our demonstration of the vasodilatory activity of low concentrations of infused nitrite in the human circulation [34], and that of many other investigators, disputes this dogma [54, 56–65]. In addition, it is now widely understood that nitrite is also derived by partial reduction of dietary nitrate by oral bacteria with subsequent absorption from the digestive system followed by concentration of nitrate back into the mouth by salivary glands; a pathway known as the nitrate-nitrite-NO cycle that is the basis of numerous studies using dietary nitrate to deliver NO to where it is needed [66].

NO is an important signaling molecule. A major pathway of NO signaling involves binding to ferrous heme proteins. For example, when NO binds to the ferrous iron of the heme protein soluble guanylyl cyclase (sGC), the protein becomes activated and produces the second messenger cyclic guanosine monophosphate (cGMP) [67–69]. This is the pathway involved in NO's action as the endothelium-derived relaxing factor (EDRF) [70]. It is produced in the endothelium by eNOS and diffuses to the smooth muscle, activating sGC which starts a cascade that relaxes blood vessels and increases blood flow. NO can also signal through protein post-translational modification by oxidation and reaction with a thiol to form a nitrosothiol (SNO) with an accessible thiol of a cysteine residue [71, 72]. For example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can be nitrosated at its Cysteine 15 residue forming SNO-GAPDH and then trans-nitrosates nuclear proteins that influence transcription [73]. In addition to acting as a vasodilator, NO reduces platelet activation and thrombosis [74–76], and reduces circulating blood cell adhesion to endothelia [77–81]. NO has also been shown, under some conditions, to improve RBC deformability [82–85]. Thus, NO could perform functions that would be beneficial to patients with SCD.

3. Nitric Oxide and Hemoglobin

One of the hints that led to the identification of the EDRF as NO was that both the activity of NO and EDRF are blunted by Hb [70]. Oxygenated Hb (OxyHb or $HbFe^{2-}O_2$) reacts with NO to form methemoglobin (MetHb or $HbFe^{3+}$) and nitrate,

$$HbFe^{2-}O_2 + NO \rightarrow HbFe^{3+} + NO_3^{-}. \quad (4)$$

This reaction, known as the NO dioxygenation reaction, is extremeley fast, being characterized by the rate constant of $6–8 \times 10^7$ 1/M1/s [86–88]. Since there is so much Hb in blood (about 10 mM in heme) directly adjacent to the endothelium where NO is made, theoretical calculations suggest that NO cannot function as the EDRF because too much NO would be scavenged via dioxygenation rather than binding to sGC in the smooth muscles [89]. However, NO can function as the EDRF (and modulate platelet activation and perform other functions) because NO reacts with Hb encapsulated in the RBC much slower (up to 1000 times) than with cell-free Hb [90–100].

Several mechanisms account for reduced NO scavenging by RBC encapsulated Hb and major mechanisms are illustrated in Figure 1. NO reacts so fast with Hb, that the observed

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rate of the reaction is limited by the rate of diffusion of NO to the RBC [91, 97, 101, 102]. Even in stopped-flow experiments, where NO and RBCs are completely mixed, NO scavenging rates are limited by the time it takes NO to diffuse to the RBC after NO just adjacent to the cell is depleted. In vivo, external diffusion is further rate-limiting due to the fact that a cell-free zone forms next to the endothelium where NO is made because RBCs tend to travel in the center of the vessel [92, 94, 103]. In addition, there is some contribution to reduced NO scavenging by RBCs due to the permeability of the RBC membrane to NO [95].

Upon hemolysis, when RBCs rupture, cell-free Hb is released and the mechanisms for reduced NO scavenging illustrated in Figure 1 are no longer applicable. That cell-free Hb scavenges NO so much faster than that encapsulated in the RBCs is largely responsible for the failure of early versions of hemoglobin-based oxygen carriers serving as blood substitutes [104]. In 2002, Gladwin and colleagues demonstrated that, despite having only about an average of 4 μM cell-free Hb (range 0 to 20 μM) compared to milimolar amounts of cell-free Hb employed as blood substitutes, NO bioavailability is blunted in patients with SCD [105]. NO-dependent increases forearm blood flow were shown to be blunted proportionally to cell-free Hb concentrations and this action was abrogated by NO inhalation which converted NO scavenging cell-free oxyhemoglobin to non-scavenging methemoglobin [105]. That patients with SCD only have 4 μM steady cell-free Hb on average that can go up to 25 μM during crisis [106] in the background of up to 10 mM RBC encapsulated Hb, has led to some skepticism regarding the role of cell-free Hb in limiting NO bioavailability in SCD [107, 108]. However, computational studies have confirmed that as little as 1 μM cell-free Hb can decrease NO bioavailability [109, 110].

4. Involvement of other mechanisms in reducing NO bioavailability

In addition to NO consumption by Hb, NO bioavailability may be impaired in SCD through NO consumption by reactions with reactive oxygen species [111]. Elevated levels of plasma activity of xanthine oxidase, which produces superoxide ($O_2^{\bullet -}$) have been detected in patients with sickle cell disease [111]. Superoxide rapidly reacts with NO to form peroxynitrite with diffusion limited kinetics ($k = 4–7 \times 10^9\ M^{-1}s^{-1}$) [112], decreasing NO bioavailability

$$NO + O_2^{\bullet -} \rightarrow ONOO^- . \quad (5)$$

NADPH oxidase has also been implicated in contributing to pathology through superoxide production [113]. In addition, reactive oxygen species can be generated through Fenton chemistry involving heme or free iron [114]. Thus, hemolysis not only can lead to increased NO scavenging through direct scavenging by Hb, but also through scavenging by reactive oxygen species that are produced due to iron release. That inhaled NO, which decreases NO scavenging by cell-free Hb through conversion to methemoglobin [105], improves vascular NO response argues for a central role of direct scavenging by cell-free Hb in low NO bioavailabilty in SCD.

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As indicated in Equation 2, arginine is a substrate for NO production by NOS. Arginine is also a substrate for arginase which converts arginine to urea and ornithine and thus decreases the availability of the NOS substrate [115]. Patients with SCD have low levels of arginine [116]. Arginase activity is very high in RBCs compared to plasma [117]. Thus, hemolysis not only releases cell-free Hb that scavenges NO, but also releases arginase which scavenges the NOS substrate arginine [118–121]. Indeed, plasma arginase activity is higher in patients with SCD than in control subjects and correlates with hemolytic rate [121]. Arginase activity has been shown to correlate with pathology and mortality [121].

5. NO-based pre-clinical and clinical trials in SCD

NO-associated pathology and its partial reversal through NO administration has been demonstrated in various murine models of sickle cell disease [122, 123]. Kaul and other showed that NO-dependent vasodilation was impaired in a model expressing human HbS and HbS-Antilles [122]. Head and colleagues showed NO inhalation improved survival in a SAD mouse model when it was exposed to extreme hypoxia [123]. Romero et al showed that dietary arginine improved reduced red cell density through inhibition of the Gardos channel in a sickle mouse model; suggesting potential of the NO pathway to decrease sickling [124]. Inhaled NO was also shown to protect a sickle mouse model from lung injury upon hypoxia/reoxygenation [125]. Head and colleagues showed that elevated sickle RBC adhesion results from a synergy between hypoxia and low NO bioavailability through upregulation of P-selectin and that NO inhalation reduced adhesion [126].

In 1997, Head, Zapol and others published a pioneering human study in which they suggested that NO inhalation increased the oxygen affinity of sickle cell hemoglobin, perhaps due to increasing HbS solubility [127]. However, Gladwin et al, also in a study conducted in humans, subsequently found that, although inhaled NO may be beneficial in other ways, it did not affect oxygen affinity [128]. Careful studies of the effects of NO affirmed that, at concentrations relevant to NO therapeutics, there is no effect on solubility and thus there is not likely to be an effect on oxygen affinity [129]. However, as mentioned above, NO inhalation was shown to improve NO bioavailability by neutralizing NO scavenging by cell-free HbS [105].

Early case reports and small placebo-controlled clinical trials showed efficacy of NO in reducing pathology in patients with SCD [130–134]. Inhaled NO significantly decreased morphine use and some pain scores in a group of 20 patients experiencing vaso-occlusive crisis [130]. Similarly, a separate study involving 18 patients found inhaled NO reduced pain during crisis and trended to decrease morphine use [134]. In the background of these positive studies it was disappointing that the results of a larger, placebo controlled study examining the effect of inhaled NO on resolution of painful crisis showed no benefit of inhaled NO compared to placebo [135].

The use of arginine to increase NO bioavailaibity in patients with SCD has also been studied in clinical and pre-clinical investigations [119, 136–138]. In a small (10 patient) study, five-day treatment with oral arginine decreased estimated pulmonary artery systolic pressure [119]. Fifteen days of arginine therapy improved microvascular function and oxidative stress

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in a sickle cell mouse model [137]. On the other hand, in a multi-center, placebo-controlled trial that involved three months of treatment, no benefits of arginine were observed in the pediatric patients studied [136]. However, the arginine dose in this study may have been too low as plasma arginine levels did not change [136]. In another pediatric study, 5 days of arginine therapy improved pain scores and opioid use in hospitalized patients [138].

Another agent that has been studied clinically is sildenafil. Sildenafil is a phosphodiesterase-5 inhibitor [139]. Phosphodietserase-5 degrades cGMP, the product of NO-activated sGC. Thus, sildenaphil effectively increases the potency of existing NO and is expected to have similar effects as increasing NO itself. Sildenaphil has been shown to improve pulmonary hemodynamics and exercise capacity in patients with pulmonary hypertension (without sickle cell disease) [140–142]. Pulmonary hypertension in sickle cell disease is associated with high morbidity and mortality [143–147] and is likely partially due to low NO bioavailability [118, 121, 148]. Thus, the walk-PHaSST trial aimed to determine if sildenaphil could improve exercise capacity in patients with sickle cell disease who had increased tricuspid regurgitation velocities [139]. After 74 patients were enrolled in this placebo-controlled, double blind study, the trial was stopped early due to adverse events, particularly hospitalization for pain. The mechanism responsible for the observed increase in hospitalization rate for painful crises due to sildenafil was not clear, but was suggested to perhaps be due to effects of cGMP on processing certain types of pain or a class effect of PDE5-inhibitors that causes back aches and limb pain, even in subjects without sickle cell disease[139].

6. Nitrite

One suggested cause for the observed lack of efficacy in the large inhaled NO trial was that sufficient systemic nitrite was not produced [135]. Compared to other agents involved in NO signaling, nitrite has some unique aspects. Nitrite's activity is potentiated in areas of hypoxia, likely due to its reaction with deoxyHb (Equation 3). Thus, nitrite can be administered systemically, but will act preferentially where it is needed in sickle cell disease – in areas of hypoxia where red cell sickling and downstream deleterious effects occur. In addition, whereas the efficacy of other agents such as S-nitrosoglutathione and Diethylamine NONOate (a direct NO donor) are blunted when RBCs are present, nitrite activity (at least in terms of inhibiting platelet activation) requires RBCs to be present [149, 150]. These data show that nitrite is bioactivated by RBCs and that nitrite is well-suited to act in blood (where RBCs are, of course, always present), producing NO activity that leads to vasodilation and inhibitions of platelet activation.

Another rather unique effect of nitrite involves its ability to protect against effects of calcium influx that results from sickling and unsickling. As mentioned above and discussed in detail elsewhere in this issue [see the review of Brugnara], calcium influx results in cellular dehydration which can increase sickling. Unlike other agents involved in NO signaling such as nitrosothiols and direct NO agents (NONOates), nitrite is protective against calcium influx-induced dehydration and associated loss of deformability [85, 149]. Nitrite also blunts exposure calcium influx induced phospholipid asymmetry that is associated with increased

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RBC adhesivity [149]. Thus, nitrite has potential to improve sickle RBC properties, particularly cellular hydration (thereby reducing sickling) and adhesivity.

Nitrite has also been shown to blunt platelet and leukocyte adhesion in a humanized transgenic sickle cell mouse model [149]. The sickle cell mouse model was fed nitrite in drinking water for three weeks and also received nitrite intraperitoneally before examining adhesion in the mesenteric circulation under local hypoxic conditions using intravital microscopy. Platelet and leukocyte adhesion was dramatically reduced compared to sickle mice without nitrite treatments [149]. In addition, nitrite treatment resulted in reduced red cell hemolysis compared to control sickle mice [149].

There have been many studies employing inhaled nitrite or nitrite obtained through dietary nitrate for a variety of conditions [65, 66, 151–164]. In many of these applications, the action of nitrite may not involve bioactivation by RBCs. However, the fact that RBCs do bioactivate nitrite and that SCD is a disease of Hb and RBCs suggest that nitrite's targeting to blood could be beneficial for patients with SCD. Interestingly, (when not polymerized) HbS is more efficient at reducing nitrite than HbA [165]. We suggest that nitrite could have pleiotropic benefits in the context of sickle cell disease as illustrated in Figure 2.

Conclusions

Substantial evidence supports the notion that the bioavailability of NO is decreased in SCD. In particular, normal response to NO in the human forearm is blunted and this effect is dependent on the concentration of cell-free Hb [105]. NO scavenging by cell-free Hb is one of and probably the major mechanism whereby NO bioavailability becomes reduced in SCD. The degree of intravascular hemolysis has been associated with increased morbidity and mortality [166]. On the other hand, some have questioned the degree to which NO scavenging and hemolysis contributes to pathology in SCD [107].

Even if NO were not substantially depleted in SCD or if it were but loss of NO bioavailability does not largely account for pathology in SCD, administration of NO activity could be beneficial in SCD. As summarized for the case of nitrite in Figure 2, the potential effects in therapeutics are pleiotropic and include vasodilation; decreased platelet activation; decreased leukocyte, platelet and RBC adhesion to the endothelium, and improved RBC hydration which would decrease sickling. In the case of nitrite, NO activity is produced preferentially in areas of hypoxia, where it is likely to be most beneficial.

Despite its promise, several clinical trials aimed at delivering NO activity have failed. Rather than refuting basic premises on which such trials are based, the neutral or negative results of these trials likely reflect the complex nature of SCD. In particular, it should be noted that although there are acute episodes, SCD is a chronic disease where damage to blood cells, the vascular system and beyond accumulates over time. Thus, rather than focusing on acute administration of NO activity, longer term chronic administration may find more success.

Acknowledgments

This work was supported by NIH grant HL058091.

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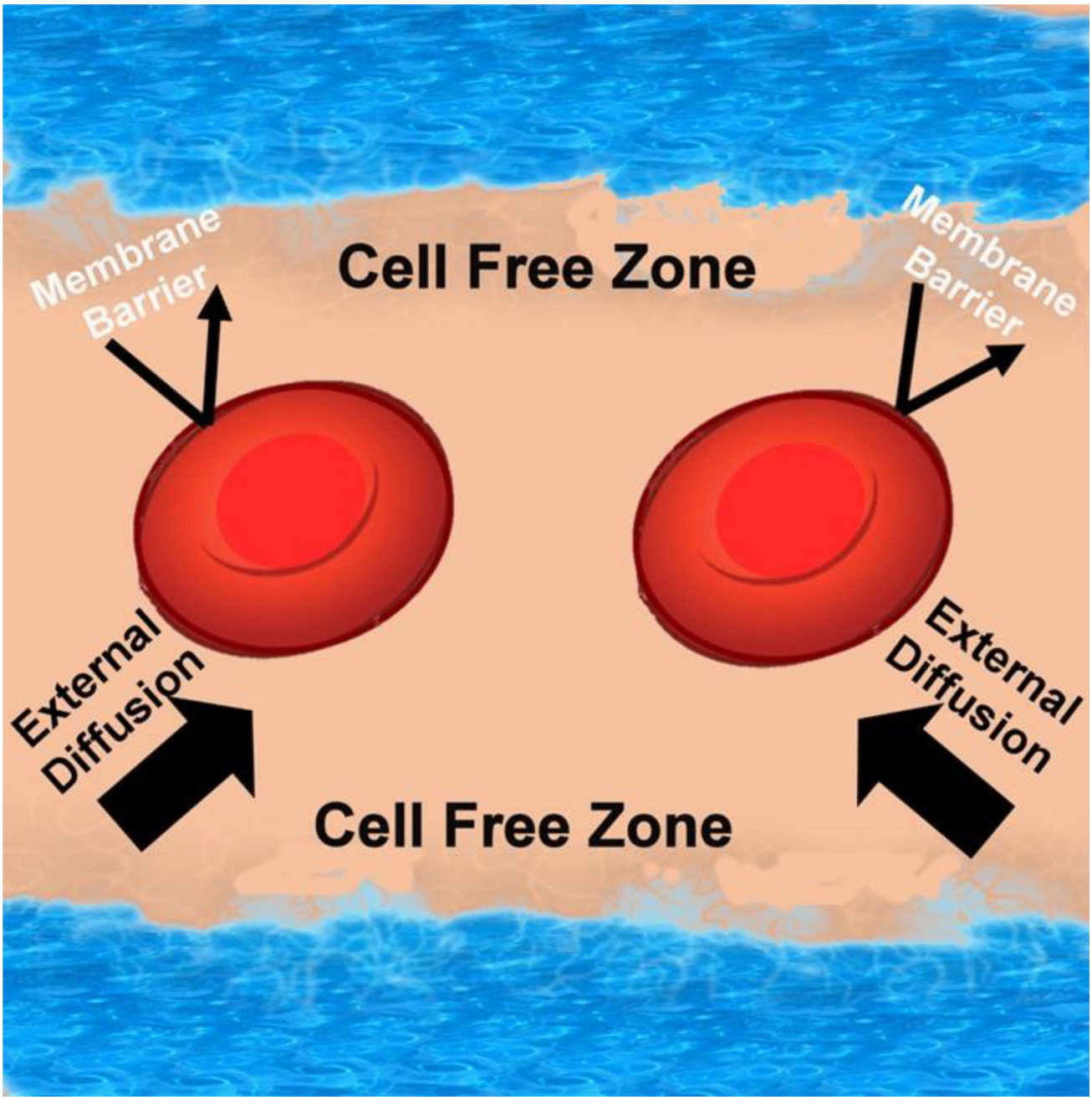

Figure 1.

Mechanisms for reduced NO scavenging by Red Blood Cells. Red blood cells are shown in side a blood vessel. The blue area represents the endothelium where NO is made from NOS and must diffuse away from the blood to reach the smooth muscles in order to effect vasodilation. NO scavenging by hemoglobin encapsulated within the RBCs in reduced due to external diffusion where the rate of scavenging is limited by the time it takes NO to diffuse to the RBCs. This time is increased due to the presence of a cell-free zone caused by red cells flowing preferentially in the center of the blood vessel, away from the endothelium.

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A finite RBC membrane permeability to NO also reduces scavenging by setting up a partial membrane barrier.

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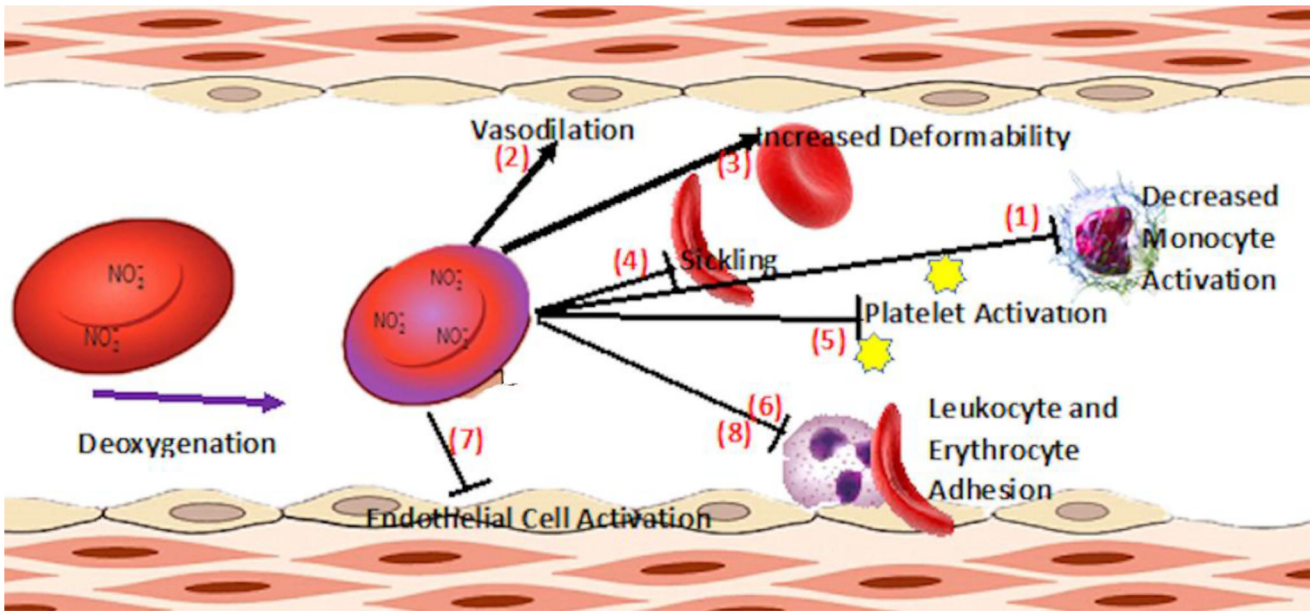

Figure 2.

Potential therapeutic action of nitrite. Upon deoxygenation, nitrite reacts with Hb. This bioactivation of nitrite by RBCs leads to (1) decreased sickling, (2) increased RBC deformability and hydration (3) increased vasodilation, (4) decreased platelet and (5) monocyte activation (6,7) decrease circulating blood cell adhesion, and decreased (8) endothelial cell activation

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Table 1

Oxidation State	Nitrogen Compound
+5	Nitrate - NO_3^-
+4	Nitrogen Dioxide - NO_2
+3	Nitrite - NO_2^-
+2	Nitric Oxide – NO
+1	Nitroxyl – HNO
0	Dinitrogen – N_2
−1	Hydroxylamine – NH_2OH
−3	Ammonia – NH_3