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Red Blood Cells as Redox Modulators in Hemolytic Anemia

Eitan Fibach and Mutaz Dana

Abstract

The oxidative status of cells, representing the balance between prooxidants and antioxidants, is involved in their normal physiological functioning, such as signal transduction, proliferation, and differentiation. When the prooxidant activity overrides the antioxidative capacity oxidative stress occurs. Chronic oxidative stress causes cytotoxicity and organ failure. As such, it is believed to play a role in various pathologies, including the hemolytic anemias. In this review, we suggest that red blood cells (RBC), in addition to their primary role as oxygen carriers, function as redox modulators. In the RBC, various systems afford it with antioxidative capacity that, in addition to balancing its own redox state, can provide antioxidative protection to the cellular and intracellular milieu throughout the body. Their vast number, mobility, occurrence throughout the body, and renewability make them good candidates for this function. A decrease in their number (anemia) or function due to oxidative stress may exacerbate the symptoms of many diseases by failing to neutralize oxidative stress. However, correcting anemia, e.g., by repeated RBC transfusions or iron supplementation, may increase the iron load, which, in turn, causes oxidative stress. This situation suggests that the status of both iron and redox should be monitored during treatment, using RBC as bioindicators.

Keywords: red blood cells, hemolytic anemia, thalassemia, oxidative stress, flow cytometry

1. Introduction

The redox status of cells is crucial for normal physiological functioning; however, when the prooxidant activity overrides the antioxidative capacity (AOC), oxidative stress occurs. Chronic oxidative stress causes cytotoxicity and organ failure. As such, it is believed to play a role in many diseases, such as cardiovascular, thromboembolic and neurodegenerative disorders, as well as aging [1].

In this review, we suggest that RBC, in addition to their primary role as oxygen and CO₂ carriers, functions as redox modulators. Various RBC systems afford them with AOC that, in addition to balancing their own redox state, can provide antioxidative protection to the cellular and intercellular milieu throughout the body. Their vast number, mobility, and occurrence throughout the body and renewability make them good candidates for this function. A decrease in their number (anemia) or function may exacerbate the symptoms of many diseases by failing to neutralize oxidative stress. However, correcting

anemia, e.g., by repeated RBC transfusions or by iron supplementation, may increase the iron load, which, in turn, causes oxidative stress. This situation suggests that the status of both iron and redox should be monitored during treatment, using RBC as bioindicators.

2. Oxidative stress and its involvement in pathology

The cellular redox status represents the balance between generation of free radicals, such as the reactive oxygen species (ROS) and reactive nitrogen species (RNS), and the ability to detoxify them or to repair their resultant damage by antioxidants, such as the reduced glutathione (GSH), the major intracellular scavenger of ROS. ROS are generated in cells mainly during energy production: In the mitochondria, about 2% of the total oxygen (O_2) consumption results in the free radical superoxide anion (O_2^-) [1]. While not particularly reactive, superoxide can act as a reductant toward divalent metal ions, mainly iron and copper, and can react with itself by spontaneous or enzymatic (e.g., by the reducing enzyme superoxide dismutase, SOD) dismutation to form hydrogen peroxide (H_2O_2). The latter is a mild oxidant, but in the presence of divalent metals, it can generate the reactive hydroxyl ($\cdot OH$) radical.

In mature RBC, which are devoid of mitochondria, the hemoglobin (Hb) is the major source of ROS generation [2]. The heme iron, which is in the Fe(II) ferrous state in the oxygenated Hb, is oxidized to the Fe(III) ferric state in metHb—a reaction that normally occurs at a rate of about 3% of the Hb per day. This process results in the production of superoxide that in turn generates hydrogen peroxide and oxygen as products of dismutation by SOD [3]. The metHb is then restored back to oxyHb by the NADH-cytochrome b5 reductase [4].

An additional pathway of oxygen to superoxide reduction is by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. Phagocytic cells, such as polymorphonuclear (PMN) neutrophils and macrophages, have an NADPH oxidase complex that generates ROS as part of the innate immune response to infection. Non-phagocytic cells contain NADPH oxidases that generate ROS, at lower levels than phagocytes, for signaling responses [5]. ROS can also arise as the indirect by-product of enzymatic activities, such as that of monooxygenases (e.g., cytochrome P450) [6].

RNS originate from the gaseous molecule nitric oxide (NO). The latter is synthesized by constitutive or inducible nitric oxide synthase enzymes by oxidation of L-arginine to L-citrulline. NO can react rapidly with the superoxide anion to form the oxidant peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2), nitroxyl (HNO), and nitrosonium cation (NO^+) (for review see [7]).

The cellular prooxidants are tightly controlled by restricting the magnitude and the location of their generation and by elaborating antioxidant mechanisms that scavenge their excess and correct their toxic consequences (for review see [1]). In addition to these intracellular antioxidant mechanisms, extracellular mechanisms function as well. For example, the blood serum contains many molecules with AOC such as bilirubin, albumin, ascorbic acid, as well as diet-derived antioxidants such as polyphenols.

Under certain conditions, excess oxidants may override the AOC and generate a state of oxidative stress. This may occur due to external factors (e.g., certain food components, air pollution, sun exposure, environmental radiation, as well as radio- and chemotherapeutic regimes) or internal factors such as various pathological circumstances (e.g., inflammation, iron overload, Hb instability). Excess ROS react quickly with bio-molecules such as the DNA, proteins, and lipids, interfering with

cellular functions. As such, even if it is not the primary etiology, oxidative stress is believed to mediate the symptoms of many diseases, such as cancer, atherosclerosis, diabetes, cardiovascular, thromboembolic and neurodegenerative disorders, as well as physiological aging [1].

3. Hemolytic anemia

We have studied oxidative stress in hemolytic anemias [8]. The anemia in these hereditary or acquired diseases is the result of augmented destruction (hemolysis) of mature RBC and their immature progenitors/precursors that is not balanced by compensatory overproduction. Among these diseases are: (I) The hemoglobinopathies—caused by mutations in the globin genes, leading to insufficient production (thalassemia) or production of aborted (sickle cell disease) globin chains [9]. (II) RBC membrane/cytoskeletal disorders such as hereditary spherocytosis, elliptocytosis, and stomatocytosis—caused by mutations in genes leading to abnormal RBC shape and propensity for hemolysis [10]. (III) Inherited enzymatic defects in RBC such as glucose-6-phosphate dehydrogenase (G6PD) deficiency and pyruvate kinase deficiency. G6PD is a key enzyme of the pentose pathway (hexose monophosphate shunt) which supplies NADPH—a reducing agent that is important for the regulation of the redox state, especially in RBC [11]. Patients with G6PD deficiency exhibit hemolytic anemia in response to infection and certain medications or foods. (IV) Paroxysmal nocturnal hemoglobinuria—a clonal disease caused by an acquired somatic mutation in the phosphatidylinositol glycan complementation class A gene. This gene encodes the enzyme responsible for the first step in the production of the glycosylphosphatidylinositol anchor, by which various proteins are linked to the plasma membrane. In this disease, the mutation occurs in a hematopoietic stem cell and is expressed in its progeny, affecting various membrane proteins including the complement (C') inhibitors: CD55 (decay-accelerating factor) which inhibits the C3 component of the C', and CD59 (membrane inhibitor of reactive lysis) which inhibits terminal C' components (C5b-9) from forming the hemolytic membrane pore [12]. This leads to hemolysis and platelet activation, leading to anemia and to venous thrombosis, respectively [13]. (V) Congenital dyserythropoietic anemias—a heterogeneous group of diseases characterized by anemia due to abnormalities of erythroid precursor cells and reduced erythropoiesis [14]. (VI) Autoimmune hemolytic anemia such as ABO mismatch transfusion reaction and severe idiopathic autoimmune hemolytic anemia—caused by autoantibodies against antigens expressed on the surface of RBC. Once formed, these antibodies bind to the surface of RBC marking them for destruction through C'-mediated lysis (intravascular hemolysis) and/or Fc-mediated phagocytosis (extravascular hemolysis). Autoimmune hemolytic anemia can occur alone, but is often seen in association with other autoimmune diseases, cancer, drug treatment, transfusion, and pregnancy. (VII) Myelodysplastic syndromes (MDS)—diverse conditions that involve ineffective production (dysplasia) of hematopoietic cells. The patients often develop severe anemia and require frequent blood transfusions. In most cases, the disease worsens, and the patient develops cytopenias due to progressive bone marrow failure. In about one third of patients, the disease transforms into acute myelogenous leukemia, usually within months to a few years.

Although oxidative stress is not the primary etiology of these diseases, except for G6PD deficiency, it mediates their symptoms, including anemia, recurrent infections, and thromboembolic complications [8]. The main causes of oxidative stress in these diseases are: (I) Degradation of abnormal Hbs in the mature RBC and their precursors (in the hemoglobinopathies), leading to the production of hemichromes

and eventually to release of heme and iron. (II) Iron overload caused by frequent blood transfusions and increased iron uptake [15]. Usually, iron uptake in the gut as well as its mobilization from storage cells, regulated by hepcidin, is downregulated by iron excess [16]. In these diseases, where the body attempts to compensate for the anemia by over production of new RBC (“ineffective erythropoiesis”), iron is in high demand. To ensure sufficient iron uptake, the developing erythroid progenitors produce factors that inhibit hepcidin production, thus overriding the regulating effect of hepcidin. (III) Iron-containing compounds (Hb and heme) which are released by intravascular hemolysis can also add to the iron load and further aggravate the hemolysis [17]. In the absence of specific mechanisms for disposal of excess iron, under these conditions iron accumulates. Iron overload increases ROS generation by catalyzing the Haber-Weiss/Fenton biochemical reactions [3, 18].

4. RBC as redox modulators

The main function of RBC is oxygen transport, for which they have evolved efficient nonenzymatic and enzymatic antioxidative systems for protection against oxidizing substances to which they are exposed. The nonenzymatic systems include reduced glutathione, thioredoxin, ascorbic acid, and vitamin E. The most important antioxidant enzymes include SOD, thioredoxin reductase/peroxiredoxin system, catalase, glutathione peroxidase, glutathione reductase, plasma membrane oxidoreductases, and the metHb reductase/NADH/glycolysis system that maintains Hb in a Fe(II)-active form [19].

Although these systems mainly serve the RBC own requirements, it seems that since they are produced in excess they can be utilized for antioxidant protection of other cells, at least under conditions of oxidative stress. This function may affect the intra- and extracellular milieu throughout the body, especially of cells in the circulation and in the perivascular tissues (endothelial cells).

Several characteristics, in addition to their extra reducing power, make RBC ideal candidates to serve as redox mediators. These include their vast number, mobility, and occurrence throughout the body. The consequence of their antioxidative activity could be oxidative damage to the RBC themselves, facilitating their erythrophagocytosis, degradation, and detoxification of their oxidized constituents by macrophages in reticuloendothelial systems, mainly the spleen and the liver. These damaged/old RBC are replaced by new RBC that are continuously formed in the bone marrow.

A “bystander” effect of cells on the oxidative status of other neighboring cells has been described previously in other circumstances of oxidative stress induced by ionizing or photoradiation [20, 21].

4.1 Proofs of the concept

The concept of the RBC protective role was first introduced by Fazi et al. [22]. They showed that RBC are able to inactivate harmful xenobiotics, including 1-chloro-2,4-dinitrobenzene, by conjugation with glutathione and suggested that it may be possible to treat xenobiotic intoxication by transfusion of GSH-loaded RBC.

Richards et al. have shown that RBC can protect endothelial cells from PMN-induced damage [23]. PMN exert their antibacterial effect by generating a burst of ROS (respiratory burst) in response to toxins released by phagocytosed bacteria. These ROS not only kill the bacteria but also damage the PMN themselves and other neighboring cells (inflammation). The respiratory burst can be reproduced

in vitro by incubating PMN with phorbol myristate acetate (PMA). In their study, ⁵¹Cr-labeled endothelial cells were incubated with PMA-triggered PMN. Damage to the endothelial cells was measured by the release of ⁵¹Cr into the incubation medium. Adding RBC to the mixture reduced the damage dose-dependently. Analyzing the RBC following the incubation, revealed reduced levels of 2,3-diphosphoglyceric acid and glutathione, and increased levels of the oxidation products malondialdehyde and metHb. These results indicated that these RBC are under oxidative stress compared with RBC incubated alone or with non-triggered PMN. The authors suggested that RBC can provide antioxidant protection to other tissues in vivo [24].

We have studied the effect of RBC on the oxidative status of other cells by measuring oxidative parameters by flow cytometry. Following pulse-labeling of cells with the probe 2'-7'-dichlorodihydrofluorescein diacetate, their fluorescence was proportional to their ROS content. The increase in their fluorescence after washing indicated their rate of generation of ROS. In our experiments, the labeled cells were incubated with RBC derived from either normal donors or patients with β -thalassemia. Normal RBC had a dose-dependent decrease effect on ROS generation, while thalassemic RBC had a much inferior effect [25].

It is well known that thalassemic RBC are under oxidative stress and contain more free iron load (the labile iron pool) than normal RBC [26]. To explore this condition on their AOC, RBC were exposed to agents that affect their oxidative stress or iron overload: normal RBC—to the oxidant hydrogen peroxide or to an iron source, ferric ammonium citrate, and thalassemic RBC—to the antioxidant N-acetyl cysteine or to the iron chelator, Desferal. The RBC were then mixed with the probe-labeled cells, and the kinetics of ROS generated by the labeled cells was monitored during incubation. The results indicated that oxidants and iron reduced the AOC of RBC (Figure 1).

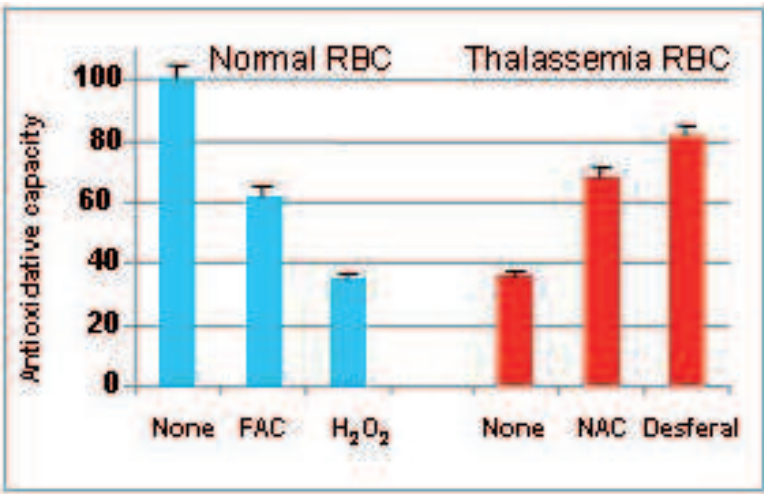


Figure 1. The effects of iron overload and oxidative stress on the antioxidative capacity (AOC) of normal and thalassemia RBC. The human myeloid leukemia HL60 cells were labeled with 2'-7'-di-chlorofluorescein diacetate, washed, and then incubated with 6×10^6 /ml RBC from normal or β -thalassemia donors ($N = 6$ each). Prior to incubation with cells, the RBC had been treated for 30 min: normal RBC with ferric ammonium citrate (FAC), 1 mM, and thalassemic RBC—with the iron chelator, Desferal, 5 mM—thus increasing and decreasing iron overload, respectively. Alternatively, normal RBC had been treated with the oxidant H₂O₂, 5 mM, and thalassemic RBCs—with the anti-oxidant N-acetyl cysteine (NAC), 5 mM—thus increasing and decreasing oxidative stress, respectively. The cells were then analyzed by a flow cytometer (FACS-calibur[®]; Becton-Dickinson, Immunofluorometry systems, Mountain View, CA, USA). The average (mean fluorescence channel) cellular green fluorescence (FL-1), reversely indicating the AOC, of 40,000 HL60 cells, was determined. In the analysis, RBC were excluded from HL60 cells by gating based on forward - and side-light scatter and fluorescence. The results indicate that the AOC of thalassemia RBC was significantly lower than that of normal RBC, and that iron and oxidants further decreased it, but it could be restored by iron chelation or antioxidants.

4.2 RBC as oxidants

Oxidative stress, being a common feature of many diseases, affects most cells of the body, including the RBC. These diseases involve RBC directly (e.g., thalassemia) or indirectly (e.g., diabetes) [27]. We have shown oxidative stress in RBC in all the hemolytic anemias [15]. Oxidative stress in RBC diminishing their own AOC, resulting in their short survival, but also reduces their ability to protect other cells. Under extreme conditions, this situation may turn the RBC into oxidative agents, rather than antioxidative agents.

4.3 Probable mechanisms involved in redox protection of RBC

Using artificial vesicles, it has been shown that while hydrogen peroxide readily crosses biological membranes, superoxide does it very slowly [28]. Vesicles made of RBC membranes allow superoxide to cross through and oxidize cytochrome c in the suspending medium within a time-frame consistent with its half-life time [29]. This transfer probably occurs via an anion channel since it was inhibited by stilbenes, which inhibit the exchange of anions across the membrane. Whether such outward flux actually occurs in intact RBC is doubtful. Since RBC contain a large amount of SOD [30], it seems unlikely that superoxide made within the RBC would escape both the spontaneous and enzymatic dismutations and diffuse across the membrane. In contrast, an inward flux could occur. The plasma contains comparatively little SOD [31], and superoxides generated outside the RBC might diffuse inward to be scavenged by the RBC-SOD. In this fashion, RBC might limit the damage inflicted by superoxides produced by blood phagocytes and vascular endothelial cells.

Similarly, RBC provide a mechanism for inactivation of free NO [32]. NO liberated from endothelium may be taken up by RBC and inactivated by oxyHb that in turn is converted to metHb, while the NO is converted to nitrate to be secreted by the kidneys.

Another RBC redox protective mechanism involves ascorbic acid (AA) (vitamin C) [33]. In humans, AA dietary intake is essential for maintaining plasma and tissue reductive capacity. It primarily functions to scavenge superoxide anion and singlet oxygen, but it also removes other ROS generated by protein-bound redox metals and xanthine oxidase. AA itself is oxidized to an AA radical and dehydro AA. Human RBC were suggested to possess a two-layered system of redox recycling of low concentrations of the AA radical under minimal oxidative stress and a backup system of recycling of large quantities of dehydro AA under increased oxidative stress. RBC accumulation of dehydro AA as a result of prooxidative conditions originates in part outside of the RBC during the two-electron oxidation of AA, which is subsequently transported reversibly in competition with glucose by the type 1 glucose transporters spanning the RBC membrane. Alternatively, dehydro AA may be lost altogether by degradation, removing a pool of potentially reversible oxidized AA. Experimental evidence suggests that recycling of AA by the RBC significantly add to the AOC of the blood [34].

Still another potential RBC redox protective mechanism is through the release of antioxidants and antioxidative enzymes (e.g., SOD and catalase) following hemolysis. We have found that hemolysate inhibits ROS generation by cells (unpublished observation). This could also occur following shedding of membrane-bound vesicles during the maturation of erythroid precursors in the bone marrow and senescence of RBC in the circulation. Both processes are enhanced in hemolytic anemias by oxidative stress [35].

Except for direct effects, RBC may affect other cells indirectly. For example, diet-derived antioxidant polyamines tend to attach to RBC membranes, resulting in a synergistic enhancement of their antioxidative activity [36].

5. Pathological significance of RBC as redox modulators

The redox-modulating activity of RBC could affect cells and their function throughout the body. We have studied their effect on platelets. Hemolytic anemia, such as thalassemia, is often associated with high incidence of thromboembolic complications (e.g., venous thrombosis and stroke) due to platelet hyperactivation and plasma hypercoagulation [37]. Platelet functioning depends on their redox state. They have an inherent ability to produce ROS by various pathways—as a by-product of the mitochondrial respiratory chain [38] and by the NADH/NADPH oxidase [for a review see [39]] produced mainly in the pentose cycle [40]. ROS, along with NO, adenosine, and prostacyclins, may play a profound role in the regulation of platelet activities [41]. Many studies demonstrated that their functioning during clot formation involves ROS; for example, platelet activators, such as thrombin, increase ROS generation [42–44].

Oxidative stress in platelets may give rise to two pathological outcomes: (I) toxicity, resulting in thrombocytopenia and bleeding and (II) hyperactivation resulting in excess clot formation leading to thromboembolic complications. Exemplifying the latter is hydrogen peroxide that stimulates their oxidative stress [45], and affects their various functions: activation by: (I) arachidonic acid and collagen [46]; (II) thrombin and ADP [47–49]; (III) tyrosine phosphorylation of the platelet $\alpha\text{IIb}\beta_3$, an independent platelet activation pathway, thereby enhancing their aggregation [50], as well as (IV) through scavenging of the platelet- or endothelium-derived NO—thereby decreasing its aggregation-inhibiting effect [51]. Superoxide can also contribute to late clot growth by increasing the bioavailability of ADP and subsequently recruiting additional platelets [49].

Since platelets do not carry known specific inherent redox pathology, it is reasonable to attribute their oxidative stress, at least in part, to continuous exposure to oxidative insults from extra platelet sources, such as their environment, i.e., the blood plasma, and neighboring cells - blood cells and the vascular endothelium. We have shown that incubation of normal platelets with plasma from thalassemia patients, rather than with normal plasma, resulted in their oxidative stress and activation [52]. Potential plasma oxidants are iron-containing compounds such as non-transferrin-bound iron, ferritin, heme, or Hb, all of which are increased in thalassemia patients [47, 53]. Incubation of platelets with iron (ferric ammonium citrate), heme (hemin or heme arginate), or Hb stimulated their oxidative stress. Moreover, addition of the iron-chelator deferoxamine to thalassemic plasma reduced its effect on the platelets' ROS [52].

Interestingly, thalassemic RBC also increased normal platelet oxidative status. In contrast, normal RBC, unless treated with oxidants, decreased it [54]. These results suggest that thalassemic RBC, having a higher than normal ROS level, mediate oxidative stress in platelets directly, probably by contact or close proximity [25]. These results are compatible with studies showing that platelets could be activated by ROS generated by neighboring cells such as RBC, neutrophils [55, 56], fibroblasts, and vascular endothelial and smooth muscle cells [39].

RBC might also affect platelets indirectly by a variety of mechanisms: (I) Release of iron-containing oxidants into the plasma [46, 57, 58], as mentioned above. (II) Release of ROS, e.g., superoxide anions, causing oxidation of low-density lipoprotein [59], which, in turn, might activate platelets [60]. (III) Exposure or shedding of phosphatidylserine moieties, which act as a procoagulant that amplifies the generation of thrombin and thus initiates platelet activation [61]. Thalassemic RBC have been shown to carry and shed higher than normal levels of external phosphatidylserine [35].

Other important function of the RBC-AOC is to scavenge and detoxify NO, an important vasodilating agent. released from the vascular endothelial cells [32] and by inflamed tissues [62].

The role of RBC as redox modulators can be compromised under pathological conditions: when their number decrease (anemia) and when their AOC is defective, both of which may co-exist in many diseases. Anemia may elevate oxidative stress by reducing the oxygen availability (hypoxia) to tissue cells and by reducing the AOC of the RBC.

Various therapeutic modalities may be used to correct anemia:

- Iron supplementation in the case of deficiency.
- Administration of erythropoiesis-stimulating agents such as erythropoietin (EPO) in cases of reduced erythropoiesis. This includes patients with chronic kidney disease where there is insufficient EPO production due to renal dysfunction, patients with malignancies during the course of chemotherapy, and patients with myelodysplastic syndrome. In most of these cases, the treatment comprises both EPO and iron supplementation.
- Blood transfusion is used in the event of acute, severe, hemorrhage, or in chronic hemolysis. An example of the latter is β -thalassemia major where patients are transfused with packed RBC every 3 weeks for their entire life.

Both transfusions and EPO have been used pre- and post-major operative procedures that are associated with severe blood loss.

All these therapeutic procedures, on one hand, increase the RBC mass and thereby, supposedly, its AOC. On the other hand, iron supplementation and transfusions might increase the iron load leading to oxidative stress in cells, including RBC, thus compromising their AOC. For example, multi-transfused thalassemic patients, with less severe anemia but higher iron overload, have lower levels of oxidative stress (ROS and lipid hydroperoxides) than un-transfused patients, with more severe anemia but lower iron overload [63]. In cardiovascular diseases, although there is ample clinical evidence for the worsening effect of anemia, RBC transfusions or EPO administration were not always effective [64–66]. As for EPO, it has been demonstrated to have an antioxidative effect on various cells, including RBC [67], and thereby might increase their AOC. The net effect of anemia and iron overload on

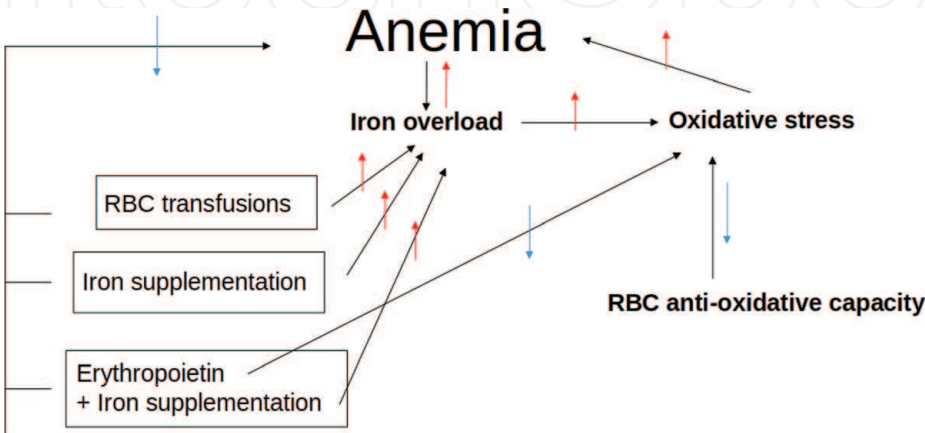


Figure 2.
The relationship among anemia, its treatment and RBC antioxidant capacity on oxidative stress. Upward red arrows indicate an increase; the downward blue arrows indicate a decrease.

oxidative stress warrants a careful study in transfused and non-transfused patients and favors continuous monitoring of the status of iron and oxidative stress during these treatments. This complex relationship is graphically summarized in **Figure 2**.

Some therapeutic protocols are used to reduce the RBC mass (hematocrit). Bloodletting (phlebotomy) is used in cases of polycythemia (erythrocytosis), either primary (polycythemia vera), familial, or secondary [68], as well as hereditary hemochromatosis—an inheritable disease characterized by iron overload [69]. The benefit of this treatment with respect to decreasing the iron load should be weighed against its potential reducing effect of the RBC-AOC.

6. RBC as redox bioindicators

The oxidative state of RBC depends on intra-RBC factors such as enzymopathology (e.g., G6PD deficiency), Hb instability (thalassemia and sickle cell disease), membrane pathology (hereditary spherocytosis), glucose metabolism [diabetes [27]], or extra-RBC factors such as in inflammation. Their oxidative state, in turn, may affect their AOC. It was suggested that RBC could be used as bioindicators of prognostic value in clinical practice [19]. They may provide a real-time monitoring of their own conditions as well as those in other parts of the body. This is potentially relevant to RBC-linked and unlinked pathologic conditions associated with oxidative stress.

6.1 Measurement of the redox status in RBC

Measurement of redox parameters in cells and in body fluids, such as the blood plasma, can be accomplished by various methods [3]. These measurements, however, are not a common practice in the clinic mainly because the methodologies are inadequate for the routine clinical laboratory. We have measured redox parameters [54, 70], including the labile iron pool [71], in RBC by flow cytometry, a common methodology in the clinical setting. Various fluorescent probes have been used. For example, ROS were measured by 2',7'-dichlorodihydrofluorescein diacetate, [72]. Following free diffusion into cells, this nonfluorescent compound is esterified and gets trapped intracellularly as 2',7'-dichlorodihydrofluorescein. ROS, mainly peroxides, oxidize it to the fluorescent derivative 2',7'-dichlorodihydrofluorescein that its cellular fluorescence is proportional to ROS generation [72].

Several points should be considered using this method: (I) Since ROS are short-lived, analyses should be performed on fresh samples. (II) The probes used are not specific to a particular ROS—a limitation that does not limit the assessment of general oxidative stress. (III) The intracellular probe content depends on the experimental settings: the concentration of probe added to the composition of the medium and the incubation conditions, such as the temperature. However, it also depends on the cellular uptake of the probe and its esterification, which depends on the different properties of cells (e.g., activated vs. inactivated, pathological vs. normal). To overcome these caveats, we have modified the protocol: Cells were pulsed with the probe, washed, and then re-incubated in probe-free medium. The kinetics of ROS generation was determined by measuring the cellular fluorescence at different times.

The method was validated by determining the effect on RBC fluorescence of the ROS-generating agent peroxide, the catalase inhibitor sodium azide, and the ROS scavenger N-acetyl cysteine. When normal RBC were compared with RBC from β -thalassemia patients, both the basal fluorescence and its kinetics were higher in the latter, confirming that thalassemic RBC were under oxidative stress.

7. Conclusions

The redox state is crucial for physiological functioning of cells, but excess reactive oxygen and nitrogen species causes oxidative stress, which is associated with many diseases, including hemolytic anemia. These anemias are characterized by accelerated destruction (hemolysis) of mature RBC and their precursors that is not balanced by compensatory overproduction. Although oxidative stress is not the primary etiology of most of these anemias, it mediates many of their symptoms. The main function of RBC is oxygen transport, for which they have evolved efficient nonenzymatic and enzymatic antioxidative systems for protection against oxidizing substances to which they are exposed. These systems serve mainly the RBC requirements, but may influence other neighboring cells as well, making the RBC antioxidative protective agents of the cellular and intracellular milieus throughout the body. Their vast number, mobility, occurrence throughout the body and renewability make them good candidates for this function. A decrease in their number (anemia) or function may exacerbate the symptoms of many diseases, including hemolytic anemias, by failing to neutralize oxidative stress. However, correcting anemia, e.g., by repeated RBC transfusions or iron supplementation, may increase the iron load, which, in turn, causes oxidative stress. This situation suggests that the status of both iron and redox should be monitored during treatment, using RBC as bioindicators and using flow cytometry multiparameter analysis.

Conflict of interest


The authors have no conflict of interests.

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