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The Transfusion Problem: Role of Aberrant *S*-Nitrosylation

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Abstract

Protein *S*-nitrosylation (the binding of a nitric oxide group to a cysteine thiol) is a major mechanism through which the ubiquitous cellular influence of nitric oxide is exerted. Disruption of *S*-nitrosylation is associated with a wide range of pathophysiological conditions. Hemoglobin exemplifies both of these concepts. It is the prototypical *S*-nitrosylated protein in that it binds, activates, and deploys nitric oxide. Within red blood cells, hemoglobin is *S*-nitrosylated during the respiratory cycle and thereby conveys nitric oxide bioactivity that may be dispensed to regulate local blood flow in the physiological response known as hypoxic vasodilation. Hemoglobin thus both delivers oxygen directly and delivers vasoactivity to potentially optimize tissue perfusion in concert with local metabolic demand. Accordingly, decreased levels of *S*-nitrosylated hemoglobin and/or impaired delivery of red blood cell-derived nitric oxide bioactivity have been observed in a variety of disease states that are characterized by tissue hypoxemia. It has been shown recently that storage of blood depletes *S*-nitrosylated hemoglobin, accompanied by reduced ability of red blood cells to induce vasodilation. This defect appears to account in significant part for the impaired ability of banked red blood cells to deliver oxygen. Re-nitrosylation can correct this impairment and thus may offer a means to ameliorate the disruptions in tissue perfusion produced by transfusion.

In conjunction with the recognition of nitric oxide (NO) as the endothelium-derived relaxing factor (1–3) was the identification of its receptor: the heme center of soluble guanylyl cyclase (sGC). (4) Binding of NO leads to increased conversion of guanosine-5'-triphosphate (GTP) to cyclic guanosine monophophate (cGMP), which in turn activates protein kinase (PK) G. These steps served as an initial explanation for how NO produced vascular relaxation though it is now clear that the majority of the functions of NO in cellular regulation are carried out independently of sGC. Indeed, it seems protein hemes do not generally mediate NO-based signaling that involve post-translational protein modifications, but rather serve to promote the requisite redox chemistry of NO. Instead, the principal protein/peptide target(s) of NO are the thiol side-chains of cysteine residues, where NO covalently binds to generate *S*-nitrosothiols (SNOs) in a process termed *S*-nitrosylation. Over one thousand proteins have been identified as targets of S-nitrosylation where activity can increase or decease in response to the addition (or removal) of NO. (5) It is increasingly apparent that the breadth of cellular activities regulated by *S*-nitrosylation may rival that controlled by phosphorylation. It is also becoming apparent that disruption of *S*-nitrosylation is an important initiator or propagator of pathologic conditions. (6). As such, resolution of aberrant *S*-nitrosylation provides an attractive therapeutic target for disease amelioration.

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The principal source of NO in mammalian cells comes from the oxidative conversion of L-arginine to L-citrulline, catalyzed by each of three isoforms of NO synthase: neuronal (nNOS/NOS1), inducible (iNOS/NOS2), and endothelial (eNOS/NOS3). (7) Direct interaction and/or sub-cellular compartmentalization of NOS with target proteins provides a basis for specificity of *S*-nitrosylation. In addition to direct modification by NO or nitrosylating equivalents, *S*-nitrosylation can also occur through thiol-to-thiol transfer of an NO group to the target protein from a small-molecular-weight *S*-nitrosothiol (most notably *S*-nitrosoglutathione, GSNO) or a SNO-protein. (8) Many SNO-proteins are in equilibrium with GSNO, as demonstrated by the increase in levels of both GSNO and SNO-proteins consequent upon genetic deletion of the principal GSNO-metabolizing enzyme, GSNO reductase. (9) Thus, denitrosylation of SNO-proteins can be mediated by transfer of the NO group to form GSNO. In addition, NO groups can be removed from SNO-proteins by de-nitrosylating enzymes including thioredoxin. (10) Thus, SNO-protein levels *in situ* reflect the operation of both nitrosylating and de-nitrosylating mechanisms.

Hemoglobin (Hb) is the prototypical *S*-nitrosylated protein in that it can bind, activate, and deploy NO as the red blood cells (RBCs) transit the circulatory system. (11) In the respiratory cycle, NO (itself or derived from nitrite or *S*-nitrosothiols) binds to heme iron (Fe^{2+} or Fe^{3+}) of deoxy, T-state Hb mainly in the venous circulation to generate HbFeNO. (12). This FeNO, which resides primarily in the beta chains of Hb, shows behavior of $Fe^{3+}NO$ - a redox-activated form of NO in equilibrium with SNO. ((11, 13) Thus, in concert with oxygenation in the lungs and the transition of Hb from T-state to R-state, the NO group can be transferred to the highly-conserved Cysβ93 residue to generate SNO-Hb. So although FeNO cannot potentially release NO for export due to re-capture by hemes present in vast excess, the heme iron in Hb provides the redox requirements for *S*-nitrosylation that generates and preserves bioactivity. The transition from high to low oxygen tension in the arterial periphery (R to T transition in Hb) promotes the release from RBCs of SNO-based vasodilatory activity. Notably, only a small fraction of the NO bound to Hb is released (i.e. transferred to acceptor thiols); the remainder is auto-captured by hemes within the beta subunit. (14, 15) Thus, in systemic arterioles, SNO-Hb will elicit increases in blood flow, whereas SNO-Hb entering the lung (in T-state) may influence ventilation-perfusion matching. (16) RBCs thereby facilitate both the uptake and delivery of oxygen. (17) Alternative proposed mechanisms for RBC-mediated hypoxic vasodilation (including the postulate that Hb acts as a nitrite reductase to generate bioactive NO (18)) are unable to satisfy the physiologically relevant features of hypoxic vasodilation. (19) These features include the response of RBCs to lowered pO_2 on a short time-scale compatible with arterial-venous transit times (seconds) and the precise gradation of vasodilatory activity linked to pO_2. In addition, no mechanism has been demonstrated through which NO generated from nitrite by Hb could escape capture and inactivation by heme iron present in overwhelming excess within RBCs. (19) Conversely, nitrite reductase can furnish NO groups that may serve as a source of SNOs. (12, 20) In sum, RBCs are the only cells to recapitulate hypoxic vasodilation and SNO-based bioactivity is the mechanism most compatible with the data.

The re-conceptualization of the respiratory cycle as a three-gas system (NO along with oxygen and carbon dioxide), (16) driven by the accumulating evidence for the importance of NO bioactivity, provides the basis for understanding why therapeutic efforts to increase the oxygen content of blood can fail to improve delivery to tissues. (21) The disconnection between oxygen content and oxygen delivery reflects the fact that tissue blood flow and not blood oxygen content is the primary determinant of oxygen delivery. (11, 16) Tissue perfusion is regulated substantially by hypoxic vasodilation, which couples metabolic demand (oxygen requirement) to local blood flow, (19, 22, 23) and it has been established that the RBC itself is a principal transducer of this response. (24–26) Under normal physiological conditions, tissue pO_2 is low, and falls further with local increases in

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metabolism, e.g., muscle activity. Thus, the release of vasodilatory NO bioactivity by RBCs sub-serves the graded increases in blood flow that are coupled to progressive decreases in the oxygen saturation of Hb. (17) RBC uptake of oxygen in the lung is optimized by analogous mechanisms that match flow to alveolar ventilation (V/Q matching), with localized hypoxic pulmonary vasoconstriction sub-serving this response to divert blood toward better-ventilated lung units. In this setting, NO trapping by hemoglobin is an important contributor to vasoconstriction. (16, 27) In sum, the binding and release of NO bioactivity in the form of SNOs is a central component of the physiologic response to local hypoxia. (16)

A thorough appreciation of the role played by SNOs in the respiratory cycle has allowed for a better understanding of an array of pathologic respiratory and circulatory conditions. Decreased levels and/or impaired bioavailability of SNO-Hb have been observed in a variety of disease states characterized by tissue hypoxemia, including pulmonary hypertension, (28) sickle cell disease, (29) diabetes, (30, 31) sepsis, (9, 32) and congestive heart failure. (33) Furthermore, several clinical (28, 34) and pre-clinical trials (35–38) have demonstrated the therapeutic benefits of restoration, maintenance, or enhancement of RBC-derived NO bioactivity. Based upon the role of NO/SNO in the delivery of oxygen by RBCs, we reasoned that the inability of banked blood to improve oxygen delivery might reflect a deficiency in SNO-Hb. (39)

Procured (i.e. donated) blood undergoes several time-dependent changes including decreased RBC flexibility, (40, 41) and increased RBC adhesiveness (42) as well as decreases in the concentration of molecular modulators of oxygen binding including 2,3-diphosphoglycerate. (43). It has been proposed that transfused blood, due to an increased affinity for oxygen combined with alterations in RBC rheology and adhesion, may exacerbate rather than correct ongoing ischemia (e.g. (44)) and thus account, at least partly, for the adverse effects of blood transfusion). However, it has been unclear how increases in oxygen affinity that are confined to a small percentage of the blood volume (transfused units) could impact overall oxygen delivery, and it remains to be shown that these biochemical or molecular measures of RBC function impacts oxygen delivery *in vivo*. Moreover, even freshly processed blood, which would not have undergone many of these storage-related biochemical changes, has been observed to decrease tissue oxygenation, (45) and very recent studies have found marked increases in mortality associated with transfusion of blood that is only a few days old. (46) Thus, it is likely that additional factors contribute to the storage-mediated alterations in RBC physiology that underlie the impairment in oxygen delivery, e.g. loss of SNO-Hb.

Donated blood is stored in an acidic-buffered isotonic solution that contains nutrients and an anti-coagulant; the pH is usually around 6.5, a condition previously shown to accelerate SNO-Hb decay. (47) Placing blood into this mixture may therefore accelerate the decline in RBC function (and venous blood is already depleted in SNO-Hb compared to arterial blood). (48) We used a combination of *in vitro* and *in vivo* techniques to determine that storage of blood leads to rapid losses in NO bioactivity, reflected by rapid losses in SNO-Hb, which are precisely paralleled by losses in the ability of RBCs to effect hypoxic vasodilation – these events are depicted in the figure. (39) (The findings were independently verified by another research team.) (49) We further showed that the defect in RBC vasodilation could be corrected by repleting SNO-Hb, raising the possibility that such an intervention might help prevent transfusion-associated ischemic morbidity. We did find that the ability to re-nitrosylate stored blood has a storage duration component in that older blood (i.e. at or approaching expiration) required additional manipulation (washing) and does not re-load SNO to the same magnitude as younger blood. Thus older blood would presumably be less capable of regenerating SNO-Hb *in vivo.*

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Modifications made to date in blood storage conditions (e.g. inclusion of nutritional supplements in the blood bag and other re-workings of the storage media, leukocyte reduction, etc.) have been directed toward maintaining RBC metabolic status and/or enhancing shelf life - none have attempted to directly restore the RBC's main function, which is tissue oxygen delivery. Similarly, although a number of storage-related deficits may at least partly resolve in viable RBCs within 24–48 h of transfusion (and 24 h *in vivo* survivability of 70% of transfused cells is the only FDA-mandated storage requirement with respect to RBC function), the observed losses in RBC SNO that occur with storage are very large relative to the amounts of NO produced *in vivo* (~1 μmol/day/70 kg). Thus, during the immediate post-transfusion period it is not anticipated that SNO levels would normalize in NO-deficient RBCs; and additional defects would further slow this process with older stored blood. Instead, stored, infused RBCs will act as overall sinks for NO, adversely affecting NO homeostasis and predisposing to vasoconstriction and ischemic insult at a time when the need is for vasodilation in the microcirculation to maintain or enhance end-organ oxygen delivery. At first pass, it may be difficult to rationalize transfusion-associated ischemic events based on depleted levels of SNO, increases in membrane rigidity or increases in oxygen affinity that characterize the small percentage of the circulating RBC pool that is contributed by transfused blood, in particular since oxygen availability is rarely limiting *in vivo*. However, increases in the affinity of Hb for oxygen are directly linked to increases in the affinity of SNO-Hb for NO, (3, 11, 16) and recent studies have reported that increases in Hb oxygen affinity are in fact associated with impaired vasodilation by RBCs. (31) Because RBCs traffic through the microcirculation in line, impaired vasodilation by a minor fraction would be expected to adversely influence oxygen delivery. In addition, there is reason to believe that infection (50, 51) and membrane rheological abnormalities (40, 41) associated with transfusion might also reflect the overall state of NO deficiency. Thus, NO deficiency may be linked to several storage defects. Conversely, enhanced oxygen delivery and restoration of NO homeostasis could result from reversing the SNO-Hb deficit at the time of transfusion.

Numerous studies across diverse patient populations indicate that transfusion is associated with a range of deleterious sequalae including pulmonary edema, renal failure, multi-organ failure, myocardial infarction, infection, increased hospital stay and death. (52–55) Notably, the Cochrane systematic review of multiple randomized trials found that liberal blood transfusion versus a more restrictive strategy is associated with a 20% increase in mortality and 56% increase in ischemic events. (56) Reports continue to accumulate describing clinical conditions where transfusion increases morbidity and mortality in adults following cardiac surgery, (46, 57) percutaneous coronary interventions, (58) and acute lung injury, (59) and in pediatric intensive care patients (60) – these are particularly striking findings in context with the increasing realization that even mild anemia is an adverse clinical predictor of outcome. (61, 62) Transfusion is also expensive. The cost of transfusion reflects not only the expense of procuring blood and ancillary expenditures (storage, cross-matching and staff wages), but also the costs incurred as a result of transfusion-related morbidity, including increased intensive care admissions and extended hospital stays. As a result, the projected cost of transfusing a single unit of packed RBCs is between $1,600 and $2,400, (63) which represents an annual expenditure of $22–34 billion in the United States. This dollar figure should be multiplied several fold to account for word-wide expenditures, and it brings into question society's return on this investment.

Severe anemia poses a significant risk to patient survival and well being (64) and transfusion may, in some situations, be lifesaving. (65, 66) But even minor anemia may adversely impact outcomes (62) while at the same time, there is little doubt about the inability of stored blood to reverse this risk or replicate the functions of native RBCs, and accumulating evidence indicates that this difference is accentuated as the storage time increases. (52–55)

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Thus, currently, raising hematocrit into the normal range by RBC transfusion is not advocated. Instead, clinical interventions are focused on identifying anemic transfusion thresholds that do not produce adverse outcomes. (64, 65) (Indeed, part of the therapeutic benefit from the anti-thrombin agent bivalirudin may well come from reducing transfusions compared to the standard anti-coagulant therapy of low-dose heparin plus Gp IIb/IIIa blockade. (67)). In principle, however, raising the hematocrit into the normal range should have beneficial effects if the oxygen-delivery function of the transfused blood could be restored. Restoration of RBC-derived NO vasoactivity through repletion of SNO-Hb appears to offer a means for blood transfusion to achieve its clinical purpose: vasodilation in the micro-circulation to maintain or enhance end-organ oxygen delivery in the anemic patient.

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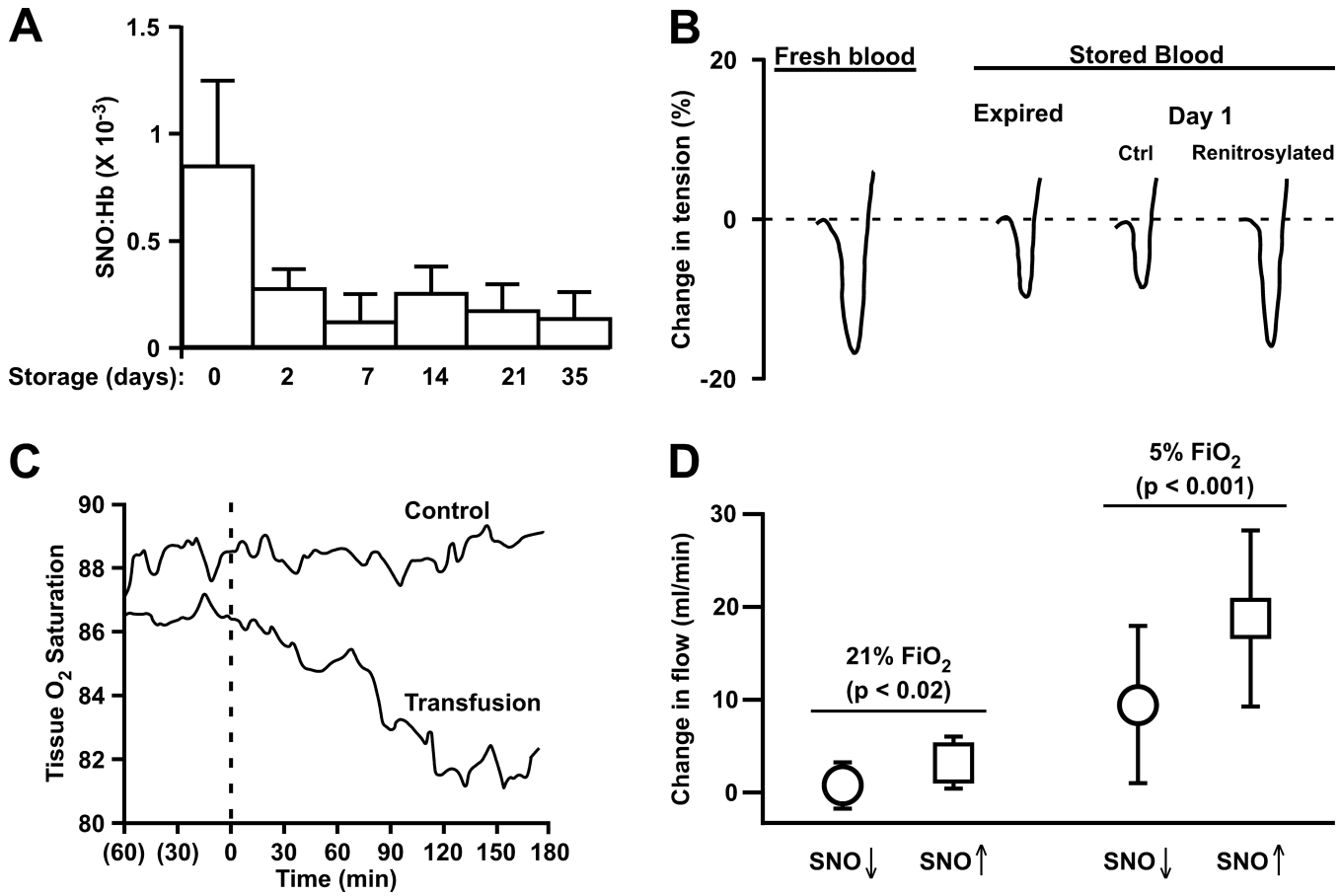

Figure.

Blood Storage diminishes *S*-nitroso-hemoglobin (SNO-Hb) concentration, hypoxic vasodilation, and oxygen delivery (modified from references 39 and 44). A) Processing of donated blood under current AABB guidelines results in the loss of most SNO-Hb within 2 days and SNO-Hb levels remain low throughout the standard storage duration; B) The loss of SNO-Hb is pronounced after one day of storage and correlates directly with diminished hypoxic vasodilation by red blood cells (RBCs) in an *in vitro* bioassay (rabbit aortic ring segments).Vasorelaxation by RBCs is restored by repleting SNO-Hb; C) Consistent with diminished hypoxic vasodilation, peripheral tissue oxygen saturation in humans (measured with a trans-cutaneous probe placed on the thenar eminence) declines following transfusion with stored blood (21 days). D) Analysis of the effects on (canine) coronary artery blood flow produced by RBC infusion reveals that increases in flow elicited by re-nitrosylated RBCs were significantly greater than those produced by SNO-depleted (stored) RBCs, and that the degree of change was greater under hypoxic (5% fractional inspired oxygen; FiO_2) than normoxic (21% FiO_2) conditions. Thus, RBCs elicit vasodilation *in vivo* that is potentiated by hypoxia and dependent on SNO bioactivity, and RBCs depleted of SNO-Hb through storage can be re-nitrosylated to enhance blood flow (i.e., cardiac oxygen delivery).