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Transition to 37°C reveals importance of NADPH in mitigating oxidative stress in stored RBCs

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The RBC storage lesion is a multiparametric response that occurs during storage at 4°C, but its impact on transfused patients remains unclear. In studies of the RBC storage lesion, the temperature transition from cold storage to normal body temperature that occurs during transfusion has received limited attention. We hypothesized that multiple deleterious events might occur in this period of increasing temperature. We show dramatic alterations in several properties of therapeutic blood units stored at 4°C after warming them to normal body temperature (37°C), as well as febrile temperature (40°C). In particular, the intracellular content and redox state of NADP(H) were directly affected by post-storage incubation at 37°C, as well as by pro-oxidant storage conditions. Modulation of the NADPH-producing pentose phosphate pathway, but not the prevention of hemoglobin autoxidation by conversion of oxyhemoglobin to carboxyhemoglobin, provided protection against storage-induced alterations in RBCs, demonstrating the central role of NADPH in mitigating increased susceptibility of stored RBCs to oxidative stress. We propose that assessing RBC oxidative status after restoration of body temperature constitutes a sensitive method for detecting storage-related alterations that has the potential to improve the quality of stored RBCs for transfusion.

Introduction

RBCs prepared for transfusion are kept at 4°C in a blood bank where they can undergo time-dependent storage lesions. These RBC storage lesions have been widely described, and they include alterations of physical and morphological properties (1–3), oxidative damage of proteins and lipids, (4–6) and metabolic changes (7, 8). However, the relationship between these RBC storage-related alterations and the well-recognized clearance of a significant percentage of RBCs in the first 24 hours after transfusion, as reviewed more than a decade ago (9), remains unclear. In 2008, Koch et al. studied more than 6000 patients undergoing cardiac surgery and showed that both short- and long-term postoperative complications were more common in patients transfused with RBCs stored for more than 14 days (10). This observation generated an ongoing debate about how long RBCs can be stored before undergoing clinically relevant storage lesions (11, 12). In a recent review, Koch et al. introduced the concept of RBC “real age,” which may be of greater biological significance than the chronological age of 42 days still accepted by the FDA (13).

RBC storage-related alterations are commonly studied with cells that have been maintained at the standard temperature of 4°C. However, stored RBCs are most often transfused at room temperature (RT) over a period of 4 hours or less. Upon entering the circulation, transfused RBCs become rapidly exposed to body temperature (37°C). The temperature transition to 37°C may exacerbate the biochemical and pathological alterations that have slowly accumulated during storage at 4°C, and this may be related to posttransfusion loss of RBCs and subsequent adverse events in vivo (14, 15).

The effect of increasing temperature on oxidative stress is of particular interest, given the essential role of redox regulation during RBC storage. RBCs use multiple redox systems to mitigate oxidative stress.

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In an oxidative environment, molecular oxygen can transform into a superoxide radical anion (O_2^-), which can then dismutate into hydrogen peroxide (H_2O_2) through the action of superoxide dismutase (SOD). H_2O_2 is consumed by several different intracellular biochemical reactions, including those involving catalase, thioredoxin/peroxiredoxin (Trx/Prx) and glutathione/glutathione peroxidase (GSH/GPx) systems (16–19). These systems that mitigate oxidative stress rely directly or indirectly on NADPH, which is generated by reduction of $NADP^+$ in the pentose phosphate pathway (PPP) of carbohydrate metabolism. A tight balance between the PPP and the Embden-Meyerhof-Parnas (EMP) pathway of glycolysis is crucial in mitigating RBC oxidative stress, and metabolic perturbation of these pathways during RBC storage has been demonstrated (20). Nevertheless, the interconnections among these redox systems, their relationships to RBC functions, and the effects of these systems on survival of transfused RBCs in circulation remain uncertain. Here we used post-storage incubation of RBCs at 37°C as an experimental approach to investigate changes to these redox systems that occur in stored RBCs after their transfusion.

Results

Post-storage incubation of RBCs at 37°C reveals decreased deformability and increased osmotic fragility. Stored RBCs have altered physical properties, indicating an impaired ability to survive in circulation through narrow channels in capillaries and splenic red pulp (21). After storage at 4°C in saline-adenine-glucose-mannitol (SAGM), our analyses of RBCs showed increased osmotic fragility and slightly reduced deformability under shear stress (Supplemental Figure 1, A and B; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.126376DS1>). Increased osmotic fragility and decreased deformability were accompanied by progressive hemolysis in the storage bags, with a maximum of 0.8% lysed erythrocytes after 6 weeks of storage (Supplemental Figure 1C). RBC integrity was only slightly affected after 2–4 weeks' storage at 4°C , but warming to 37°C markedly increased osmotic fragility and decreased deformability after 4 hours, and the effect was even greater after 20 hours (Figure 1, A and B). Osmotic fragility achieved a plateau by 18 hours and remained stable with incubation longer than 20 hours (Supplemental Figure 2). Temperature sensitivity was acquired during the 4°C storage duration for deformability (weeks 4 and 6), whereas it was independent of storage duration for osmotic fragility (Figure 1, A and B). In fact, temperature sensitivity was detected in freshly collected samples prior to erythrocyte concentrate preparation (Supplemental Figure 3). Hemolysis rates were not affected by incubation for 20 hours at 37°C (Supplemental Figure 4).

Post-storage incubation of RBCs at 37°C reveals an inability to mitigate oxidative stress. The extent of aminotriazole-mediated (AT-mediated) inhibition of catalase activity increased when RBCs were incubated at 37°C compared with 4°C (Figure 1C), indicating increased formation of H_2O_2 at elevated temperature. The increased formation of H_2O_2 at 37°C indicated the importance of antioxidant defenses that protect RBCs during storage. Prx2, a thiol protein and major H_2O_2 -degrading enzyme in RBCs, showed increased dimerization (a surrogate measure of increased activity in H_2O_2 degradation) during storage at 4°C , with values mostly less than 15% and wider donor variability after longer storage periods (Supplemental Figure 1D, Supplemental Figure 5, and full uncut gels). Intracellular NADPH remained stable during storage at 4°C . Total NADP(H) increased moderately after 2 weeks but returned to baseline at week 6 (Figure 1E), with the majority present in the reduced form on week 6 (Figure 1F), suggesting adaptation to storage-induced oxidative stress. Nevertheless, RBC redox systems appeared greatly stressed upon incubation at 37°C . After 6 weeks of storage, a 20-hour incubation at 37°C led to 60% dimerization of Prx2 (Figure 1D) and a decrease in total NADP(H) (Figure 1E), with a dramatic loss of NADPH (Figure 1F). The decrease in NADPH was confirmed by both an enzymatic cycling method and HPLC (Supplemental Figure 6, A and B). The observed decrease in NADP(H) at 37°C is consistent with a previous report of NADP(H) modulation upon RBC storage at 25°C (normal RT) (22).

The increased formation of H_2O_2 upon temperature elevation suggested that RBC dysfunction is related to an inability to mitigate increased oxidative stress. In order to test this hypothesis, fresh RBC samples were stored up to 48 hours in the presence of copper/ascorbate as a source of exogenous oxidative stress. Molecular oxygen oxidizes cuprous (Cu^{1+}) ions to cupric (Cu^{2+}) ions with the formation of O_2^- , which dismutates to H_2O_2 , while ascorbate reduces Cu^{2+} to Cu^{1+} . The observed increase in AT-mediated inactivation of catalase in the presence of copper/ascorbate during 24-hour incubation confirmed enhanced production of H_2O_2 (Figure 2A). Copper/ascorbate treatment of RBCs led to increased Prx2 dimerization within 1 hour (Figure 2B) and a depletion of total NADP(H) (Figure 2C) and decreased NADPH/NADP⁺ ratio due

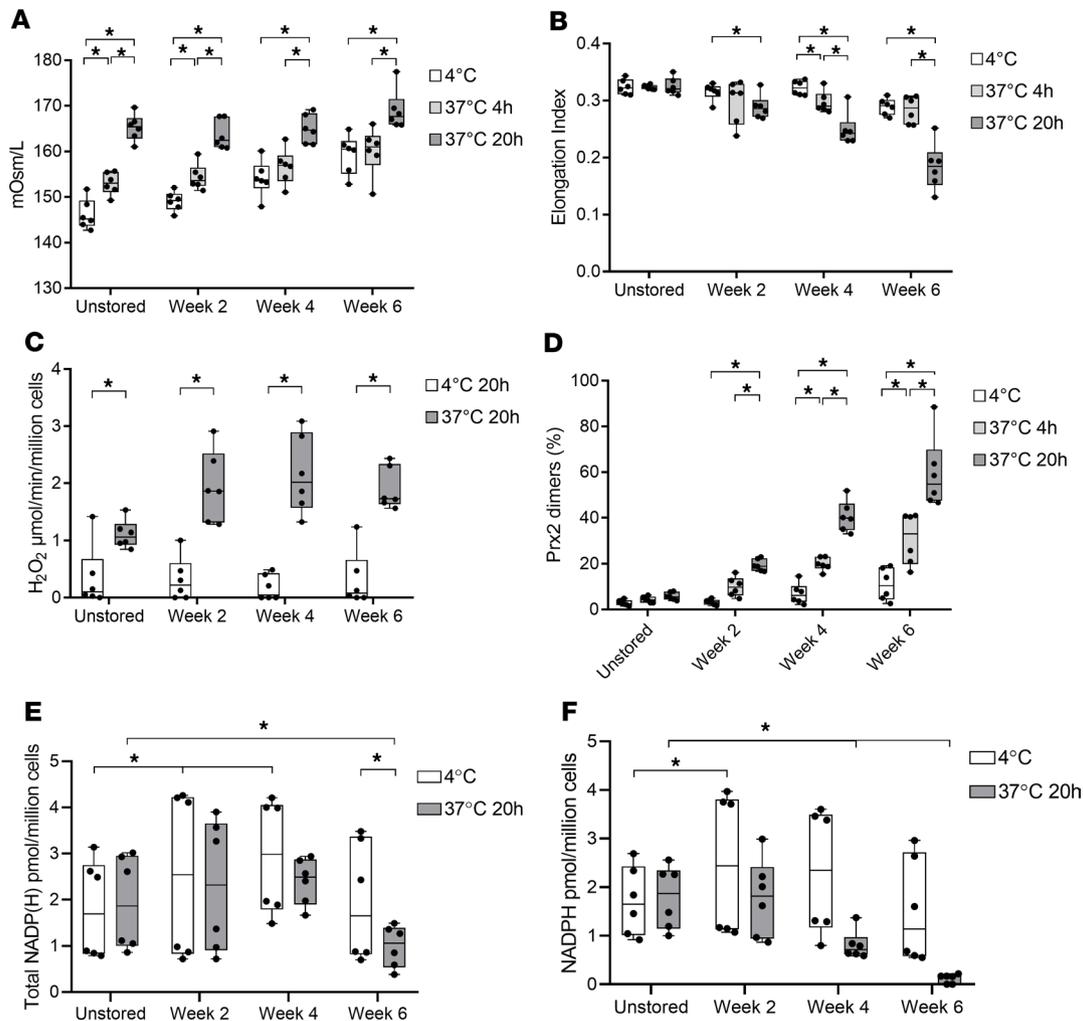


Figure 1. Effects of incubation at 37°C on physical properties, Prx2 dimerization, and NADP(H) content of RBCs previously stored at 4°C. (A) Osmotic fragility based on solution osmolality leading to 50% hemolysis ($n = 6$); and (B) elongation index (measure of RBC deformability) ($n = 6$) at 0, 2, 4, and 6 weeks of storage at 4°C before (white) and after warming to 37°C for 4 hours (light gray) or 20 hours (dark gray). (C) Hydrogen peroxide formation as described in Methods during 20 hours at 4°C or 37°C (5% hematocrit) after storage for 0, 2, 4, or 6 weeks at 4°C ($n = 6$). (D) Percentage Prx2 dimerization ($n = 6$) at 0, 2, 4, and 6 weeks of storage at 4°C before (white) and after warming to 37°C for 4 hours or 20 hours. (E) Total NADP(H) and (F) NADPH determined by enzymatic cycling at 0, 2, 4, and 6 weeks of storage at 4°C before and after warming to 37°C for 24 hours ($n = 6$). Box plots show median, 25th and 75th percentiles (box), and minimum/maximum values (whiskers). * $P \leq 0.05$, repeated-measures 2-way ANOVA. See Supplemental Figure 5 and full uncut gels.

to loss of NADPH (Figure 2D). Increased oxidative stress in RBCs led to deterioration of RBC functional properties, with a progressive increase in osmotic fragility and hemolysis over a 48-hour period (Figure 2, E and F). No effect on deformability was observed (data not shown). Thus, exposure of freshly processed RBCs to oxidative stress in the form of copper/ascorbate recapitulated most of the biochemical and pathological changes found in RBCs stored at 4°C and then warmed to 37°C. Together, these results identify osmotic fragility as a sensitive measure of diminished capacity to mitigate oxidative stress (23–25), thereby representing an early marker of the RBC storage lesion.

Degradation of RBC physical properties at febrile body temperature. To assess the effect of transfusion in febrile patients, we warmed stored RBCs to 37°C or 40°C for 20 hours. The increase from 37°C to 40°C aggravated the deleterious effects on RBCs that had been stored for as little as 2 weeks. Osmotic fragility increased further while deformability decreased further after 20 hours at 40°C as compared with 20 hours at 37°C (Figure 3, A and B). These results suggest that febrile temperatures are particularly harmful to RBCs, even in an *in vitro* environment without the shear forces associated with blood flow. Thus, compared with normal body temperature, a febrile state is likely to exacerbate RBC loss in the first 24 hours after transfusion (26).

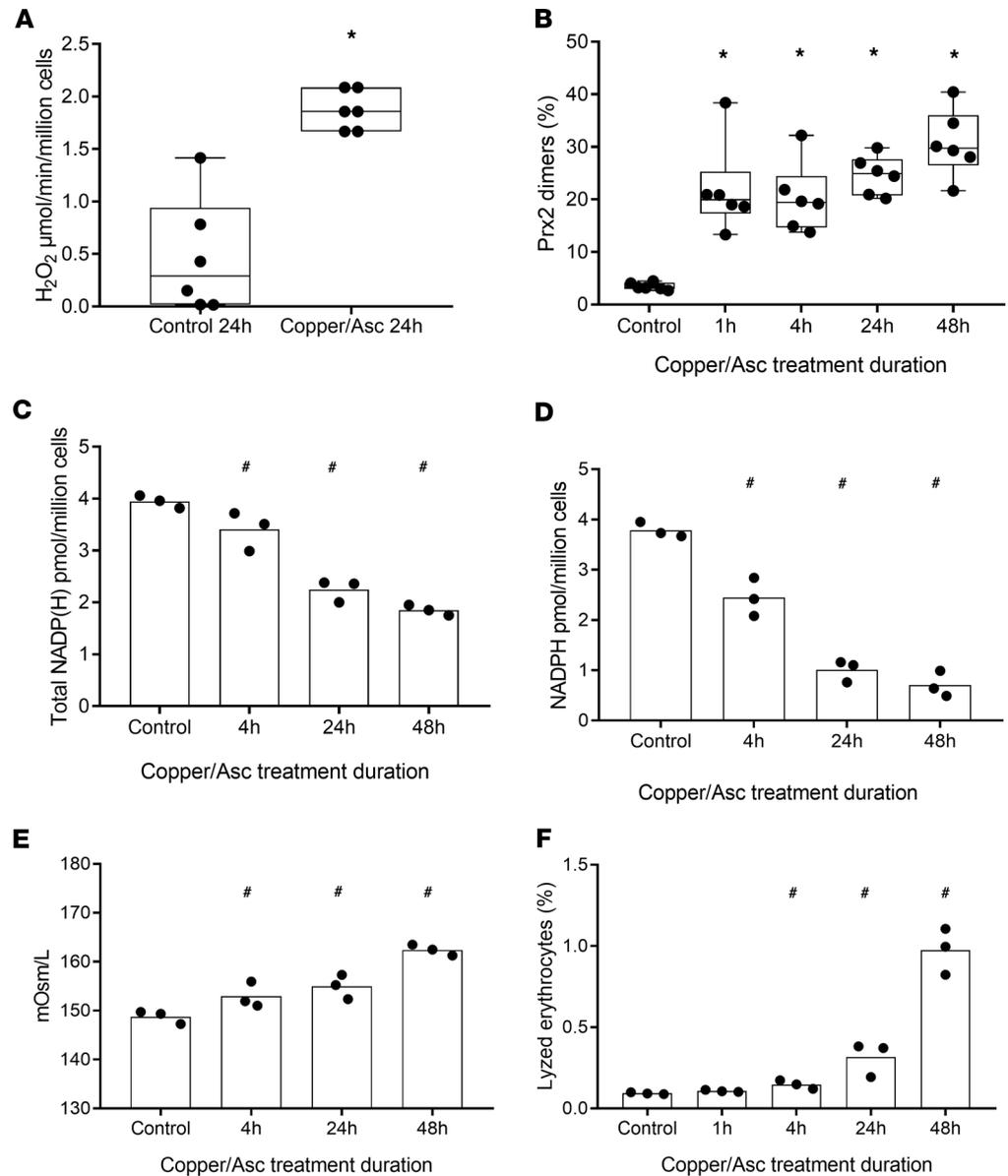


Figure 2. Effects of copper/ascorbate-induced oxidative stress on physical properties, Prx2 dimerization, and NADP(H) content of freshly processed RBCs. (A) H₂O₂ formation by RBCs (5% hematocrit) during 24 hours at 4°C in the absence (Control) or presence of copper/ascorbate (Asc) in fresh blood samples ($n = 6$). (B) Percent Prx2 dimerization ($n = 6$) in fresh blood samples exposed to copper/ascorbate for 0 (Control), 1, 4, 24, or 48 hours. (C) Total NADP(H) and (D) NADPH by enzymatic cycling in fresh blood samples exposed to copper/ascorbate for 0, 1, 4, 24, or 48 hours at 4°C ($n = 3$). (E) Osmotic fragility based on solution osmolality leading to 50% hemolysis ($n = 3$) and (F) percent lysed erythrocytes ($n = 3$) in fresh blood samples exposed to copper/ascorbate for 0, 4, 24, or 48 hours at 4°C. Box plots show median, 25th and 75th percentiles (box), and minimum/maximum values (whiskers). Bars show mean values. * $P \leq 0.05$, repeated-measures 1-way ANOVA in comparison to control; # $P \leq 0.05$, Mann-Whitney nonparametric test.

Oxygen displacement from hemoglobin prevents Prx2 dimerization but not increased osmotic fragility associated with warming to 37°C. We next sought to examine the relationship between oxidative stress and RBC function by decreasing oxidative stress during RBC storage. As autoxidation of oxyhemoglobin can be a major cause of oxidative stress in RBCs by forming O₂⁻ and H₂O₂, (27) we displaced O₂ bound to hemoglobin with CO, which has an affinity for hemoglobin that is 200 times that of oxygen. Treatment of freshly collected RBC suspensions with CO led to the expected change in absorption spectrum (Figure 4A), indicated by the increase in the 541-nm/577-nm ratio from 0.97 to 1.19, which was maintained at 1.15 after 2 weeks. CO treatment also led to a decrease in the formation of H₂O₂ after 20 hours at 37°C in freshly processed RBCs, although this decrease was lost after 2 weeks of storage (Figure 4B). Conversion of oxyhemoglobin

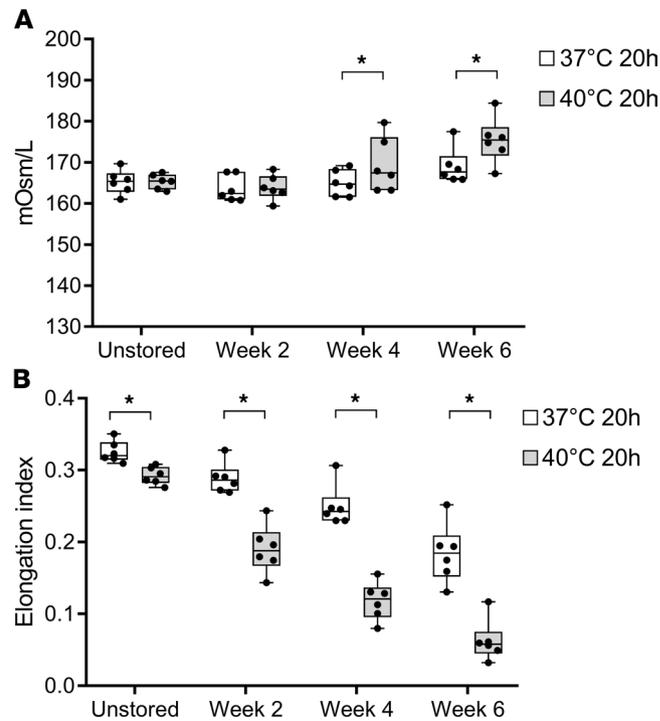


Figure 3. Effects of warming stored RBCs to 37°C versus 40°C. (A) Osmotic fragility based on solution osmolality leading to 50% hemolysis ($n = 6$); and **(B)** elongation index ($n = 6$) at 0, 2, 4, and 6 weeks of storage at 4°C after exposure to 37°C for 20 hours (white) or 40°C for 20 hours (gray). Box plots show median, 25th and 75th percentiles (box), and minimum/maximum values (whiskers). * $P \leq 0.05$, repeated-measures 2-way ANOVA.

to carboxyhemoglobin completely abrogated Prx2 dimerization, an effect that persisted at 2 weeks (Figure 4C). Despite prevention of Prx2 dimerization, displacement of hemoglobin-bound oxygen with CO did not prevent an increase in RBC osmotic fragility after 20 hours at 37°C (Figure 4D) or at 4°C (data not shown). Thus, Prx2 dimerization was prevented by oxygen displacement from hemoglobin by CO, but this treatment had no effect on RBC osmotic fragility induced by incubating at 37°C.

Modulation of the PPP provides protection of RBC function. The observed dissociation between Prx2 dimerization and osmotic fragility suggested that redox processes other than hemoglobin autoxidation and H_2O_2 metabolism by Prx2 are important during RBC storage. NADPH is a major cellular reducing species provided by the oxidative branch of the PPP. Activation of the PPP at the expense of the EMP has been described as a major aspect of the metabolic switch that occurs in RBCs during storage (20). However, a direct relationship between modulation of the PPP and RBC function has not been established.

To support and enhance PPP activity, we supplemented the standard SAGM storage solution with an RBC processing solution, containing 20.8 g/L sodium phosphate, 26.8 g/L inosine, 11 g/L sodium pyruvate, and 680 mg/L adenine (PIPA rejuvenation solution; Rejuvesol Red Blood Cell Processing Solution, Zimmer Biomet; refs. 28, 29), that is available for clinical use in the United States. Freshly processed RBCs were treated with copper/ascorbate after addition of 15% (vol/vol) PIPA solution. The added PIPA solution prevented copper-induced osmotic fragility and hemolysis (Figure 5, A and B). The PIPA solution also reversed storage-induced osmotic fragility when added for a rejuvenation of 1 hour, as well as prevented osmotic fragility during the first 2 weeks of storage when added at 15% (vol/vol) prior to storage to the total volume of RBCs suspended in SAGM (Figure 5, C and D). This effect was maintained for 6 weeks when doubling the PIPA to 30% (vol/vol) (Figure 5D) or when replenishing with fresh PIPA at 15% (vol/vol) after 3 weeks of storage (Figure 5E). Of note, addition of as much as 40% SAGM (vol/vol) to the total volume of RBCs suspended in SAGM did not affect RBC osmotic fragility. PIPA addition prior to storage also partially prevented hemolysis during storage (Figure 5F). Addition of 15% PIPA (vol/vol) prior to storage partially restored the NADP(H) content and prevented oxidation of NADPH after 6 weeks of storage followed by 20 hours at 37°C, as shown both by the enzymatic cycling method and HPLC (Figure 5, G and H, and Supplemental Figure 6, C and D). These results reveal the central role of the PPP in maintaining

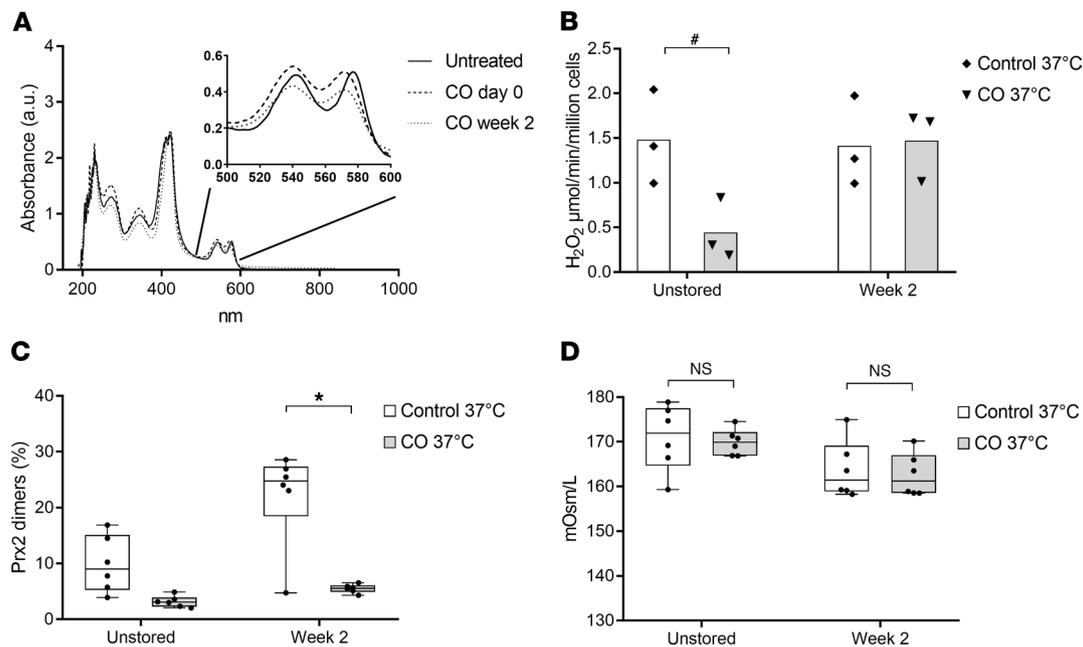


Figure 4. Effects of oxygen removal by CO purging on oxidative stress and osmotic fragility after warming to 37°C of RBCs previously stored for 2 weeks. (A) Hemoglobin absorption spectra (OD) of untreated (solid line) or CO-treated RBCs analyzed 1 hour (dashed) or 2 weeks (dotted) after treatment. (B) Formation of H₂O₂ by RBCs without (diamonds) or with CO treatment (triangles). RBCs were stored for 0 or 2 weeks at 4°C, followed by 20 hours at 37°C at 5% hematocrit (*n* = 3). (C) Percent Prx2 dimerization (*n* = 6) and (D) osmotic fragility based on solution osmolality leading to 50% hemolysis (*n* = 6) in untreated (white) or CO-treated (gray) blood samples. RBCs were stored for 0 or 2 weeks at 4°C, followed by 20 hours at 37°C. Box plots show median, 25th and 75th percentiles (box), and minimum/maximum values (whiskers). Bars show mean values. **P* ≤ 0.05, repeated-measures 2-way ANOVA; #*P* ≤ 0.05, Mann-Whitney nonparametric test.

the redox balance in stored RBCs and indicate that maintenance of NADPH during storage of RBCs has the potential to increase the life span and functional qualities of RBCs.

In order to understand the contribution of the different components of the PIPA solution, we supplemented fresh blood with a solution of either inosine or pyruvate alone, at concentrations matching those in the PIPA solution. Both inosine and pyruvate showed an improvement in copper-induced osmotic fragility, and a clear decrease in copper-induced hemolysis. These responses were only partial compared with the results obtained with the PIPA solution (Figure 6, A and B). Inosine and pyruvate slightly improved storage-induced osmotic fragility for the first 2 weeks, but to a lesser extent than the PIPA solution (Figure 6C).

Discussion

The incubation at 37°C of RBCs stored at 4°C simulates transfusion-associated transition to normal human body temperature (25, 30). The physiological relevance for transfused patients of the minor biochemical and cellular alterations that may occur in RBCs taken directly from 4°C storage may be difficult to discern. However, these changes underlie more extensive lesions that are revealed upon short-term incubation at 37°C. In part, the changes we observed may have been exacerbated in our *in vitro* experimental system compared with transfused RBCs *in vivo* due to the restrictions of a closed system, deficiencies of metabolic substrates, and limited buffering capacity *in vitro*. Nevertheless, storage induces changes to RBCs that are likely to make them more susceptible to damage associated with the relatively abrupt transition to *in vivo* conditions including increased temperature following transfusion. Our results showed temperature-related detrimental changes that were even more pronounced upon incubation at 40°C, suggesting that temperature reduction in febrile patients may also be investigated as a strategy for improved transfusion outcomes.

During cold storage, RBCs are exposed to oxidative stress (4–6). NADPH is a major reducing agent in RBCs that provides reducing equivalents to Trx reductase (TrxR) that are used to regenerate Trx. Trx, in turn, regenerates Prx2 that degrades H₂O₂ (18). NADPH also protects catalase from inactivation during metabolism of H₂O₂. While NADPH concentrations were maintained during 6 weeks' storage of RBCs at 4°C, suggesting an active PPP (20), increasing the temperature to 37°C decreased the NADPH/NADP⁺

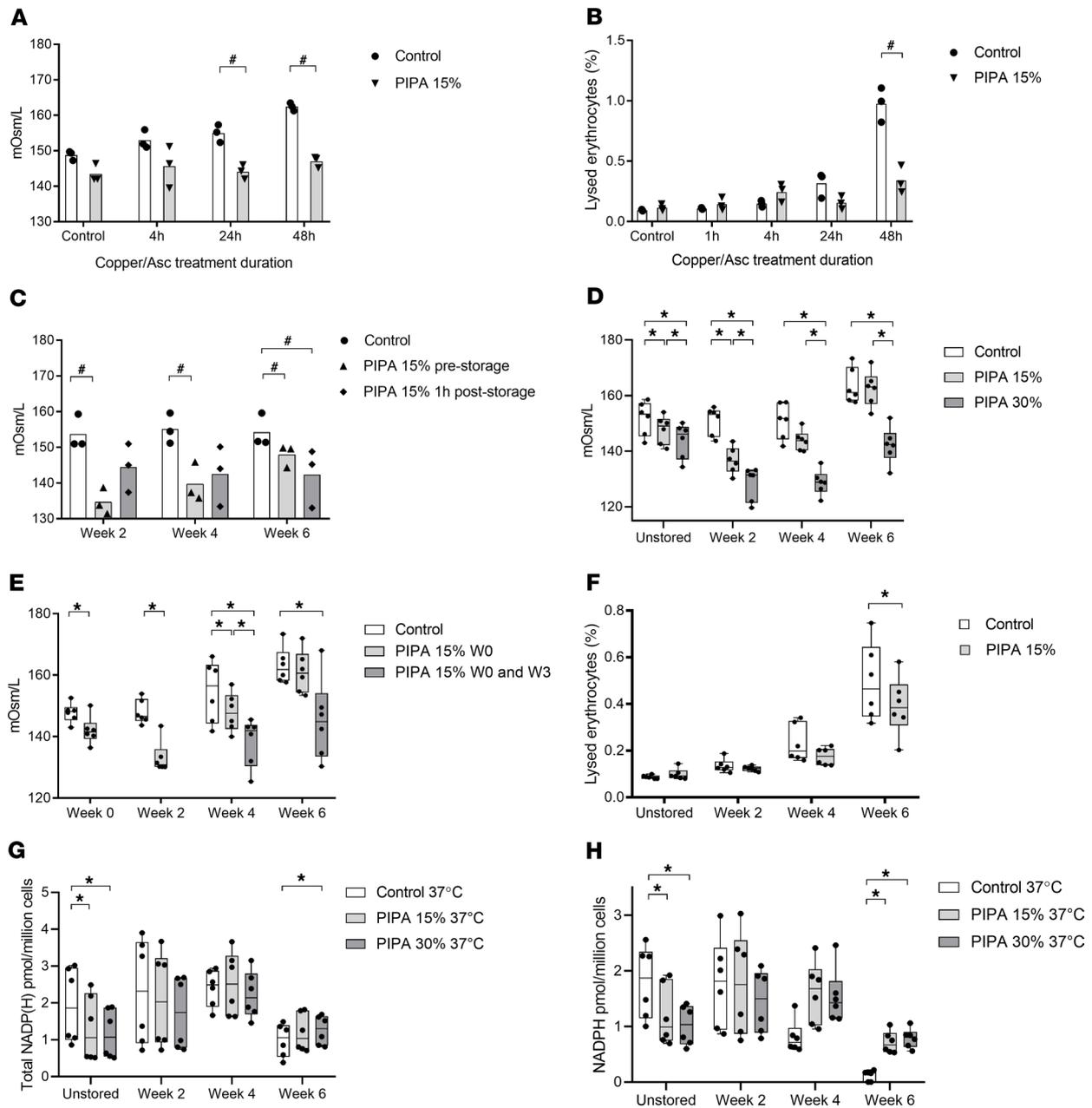


Figure 5. Effects of PIPA on physical properties, Prx2 dimerization, and NADPH content of RBCs stored at 4°C prior to challenge with copper/ascorbate treatment or warming to 37°C. (A) Osmotic fragility based on solution osmolality leading to 50% hemolysis ($n = 3$); and (B) percent lysed erythrocytes ($n = 3$) in untreated RBC samples (circles) or samples treated (triangles) with 15% PIPA solution (sodium phosphate, inosine, sodium pyruvate, and adenine), followed by copper/ascorbate treatment for 0 (Control), 4, 24, or 48 hours at 4°C. (C–E) Osmotic fragility based on solution osmolality leading to 50% hemolysis in untreated RBC samples (white), samples treated with 15% PIPA solution on day 0 (light gray), (C) samples treated during 1 hour with 15% PIPA solution after storage (dark gray) ($n = 3$), (D) samples treated with 30% PIPA solution on day 0 (dark gray) ($n = 6$), or (E) samples treated with 15% PIPA solution at week 0 and week 3 (dark gray) ($n = 6$), and stored at 4°C for 0, 2, 4, or 6 weeks. (F) Percent lysed erythrocytes ($n = 6$) in untreated RBC samples (white) or samples treated with 15% PIPA solution on day 0 (gray) and stored at 4°C for 0, 2, 4, or 6 weeks. (G) Total NADP(H) and (H) NADPH determined by enzymatic cycling in untreated RBC samples (white) or samples treated with 15% (light gray) or 30% (dark gray) PIPA solution on day 0 and stored at 4°C for 0, 2, 4, or 6 weeks, followed by 20 hours at 37°C ($n = 6$). Box plots show median, 25th and 75th percentiles (box), and minimum/maximum values (whiskers). Bars show mean values. * $P \leq 0.05$, repeated-measures 2-way ANOVA; # $P \leq 0.05$, Mann-Whitney nonparametric test.

ratio while substantially increasing H_2O_2 formation and Prx2 dimerization. Enzymatic cycling and HPLC assays showed similar patterns in NADPH changes during storage, but a difference in absolute intracellular NADPH quantities between the 2 methods was observed (Supplemental Figure 6). One likely explanation for the difference in these assays is that the sample extraction and separation in the HPLC method may have removed a cellular component that contributed to absorbance in the enzymatic method.

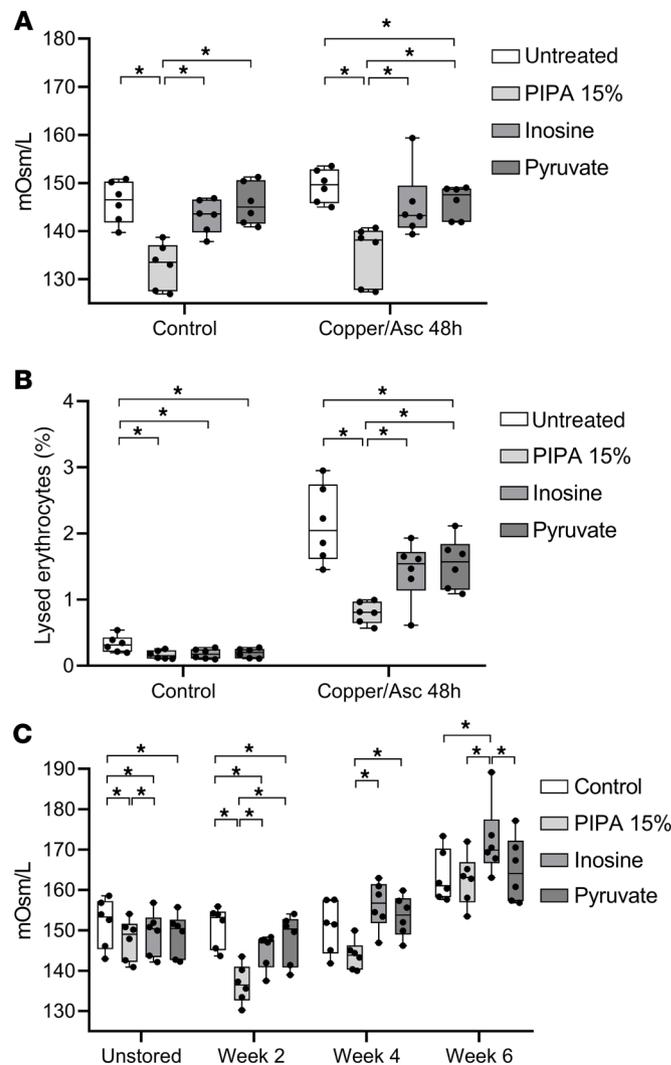


Figure 6. Effects of adding inosine or pyruvate on physical properties of RBCs stored at 4°C. (A) Osmotic fragility based on solution osmolality leading to 50% hemolysis ($n = 6$) and (B) percent lysed erythrocytes ($n = 6$) in untreated RBC samples or samples treated with 15% PIPA solution, 15% inosine solution, or 15% pyruvate solution, followed by copper/ascorbate treatment for 0 (Control) or 48 hours at 4°C. (C) Osmotic fragility based on solution osmolality leading to 50% hemolysis in untreated RBC samples, samples treated with 15% PIPA solution, 15% inosine, or 15% pyruvate and stored at 4°C for 0, 2, 4, or 6 weeks ($n = 6$). Box plots show median, 25th and 75th percentiles (box), and minimum/maximum values (whiskers); $*P \leq 0.05$, repeated-measures 2-way ANOVA.

Repeated CO purging to displace oxygen from hemoglobin and remove it from stored RBCs prevented Prx2 dimerization. However, this oxygen removal did not prevent increased RBC osmotic fragility. With copper/ascorbate oxidative treatment, CO purging was similarly unable to prevent the oxidation of NADPH (Supplemental Figure 7). These results suggest a limited role for Prx2 in preventing oxidant-related damage in RBCs stored at 4°C, which is consistent with studies of inhibitory control of Prx2 dimerization in response to various peroxide stimuli (18, 31). Likewise, it appears that reactive oxygen species derived from hemoglobin autoxidation are unlikely to play a significant role in increased osmotic fragility of stored RBCs and that other reactive oxygen species are at play. Reactive oxygen species in stored RBCs could be from multiple sources, such as NADPH oxidase (32), heme and iron released from hemoglobin (33), plasma membrane NADH and ascorbate oxidoreductase activity (34, 35), as well as a decrease in the antioxidants ascorbate (36) and NADPH. The PPP is active during RBC storage (20, 25, 30). The balance between EMP and PPP is modulated by the oxygenation status of hemoglobin, through competition of deoxyhemoglobin and glycolytic enzymes for the cytosolic domain of the anion transporter band 3. The displacement of the glycolytic enzymes leads to their activation when RBCs are stored under hypoxic conditions resulting from argon purging (20, 37). However, in stored RBCs with oxygen removal by CO purging, carboxyhemoglobin, like oxyhemoglobin, does not bind to band 3 and therefore does not affect the EMP/PPP balance by glycolytic enzyme displacement and activation (38, 39).

NADPH reducing equivalents are used for most antioxidant systems in RBCs. The nucleotide salvage metabolism recycles adenine and inosine, allowing the regeneration of NAD⁺ and NADP⁺, and NADP⁺ is subsequently reduced to NADPH by the PPP (22, 40). A decline in adenine and subsequent accumulation of its deaminated form, hypoxanthine, has been frequently noted as a marker

of alterations in stored RBCs (41, 42), and was confirmed in our experiments (data not shown). The PIPA processing solution provides additional sources of adenine and inosine that may be used for maintaining NAD⁺ and NADP⁺ nucleotides. In addition, inosine and adenine promote the PPP by providing pentose phosphate, and to a lesser extent the EMP pathway by providing a source of glyceraldehyde-3-phosphate (7, 43–45). The increased EMP activity allows for repletion of 2,3-DPG, which promotes hemoglobin-oxygen dissociation in peripheral tissues, and is depleted during RBC storage. Production of 2,3-DPG is further supported by the addition of pyruvate and consequently increased availability of NAD⁺ by conversion of pyruvate to lactate (29, 46, 47). Supplementation of RBCs stored at 4°C with the PIPA solution partially preserved NADPH after raising the temperature of stored RBCs to 37°C, indicating enhanced activity of the PPP. In the presence of the PIPA solution, osmotic fragility was improved during the first 2 weeks of storage, but protection was lost thereafter. Inosine or pyruvate also appeared to have a transient effect on RBC functional properties. Consumption of adenine, inosine, and pyruvate over time might account for this loss. Limited effects of the PIPA solution on osmotic resistance have been described (48) and are consistent with a metabolic study that showed the inability of RBCs to consume adenine after 18 days of storage (49). This reveals the complexity of RBC functional lesions, which cannot be solely attributed to NADP(H) loss and is likely to require a more diverse combination of metabolic enhancers. Preliminary experiments in our hands indicated that inclusion of various amounts of plasma at the time of red cell storage reduced osmotic fragility, indicating the need for a multidimensional metabolic intervention to improve RBC function.

Incubation at 37°C revealed the presence of a population of cells highly susceptible to osmotic lysis already present in unstored RBC samples (25). It is likely that this subpopulation of vulnerable RBCs accounts for some of the *in vivo* hemolysis observed 24 hours after transfusion (23). This susceptibility to osmotic lysis, which increases with time of storage, was not associated with increased RBC deformability. Therefore, we believe that oxidative stress does not directly impact the mechanical properties of the RBC membrane, but rather has an impact on the maintenance of ion gradients, thereby increasing RBC susceptibility to lysis. These results raise the question of whether it might be preferable to remove the subset of most fragile RBCs before transfusion rather than trying to salvage them. Approaches to physically remove susceptible RBCs might be investigated as a means of improving the quality of stored RBCs for transfusion.

Methods

Blood collection and storage. Blood was collected from 34 healthy donors at the Transfusion Center of the University Hospital of Geneva (23 females, 11 males, age 41 ± 15, mean ± 1 SD) in CompoFlow blood bags (CQ32250 Fresenius Kabi) containing citrate-phosphate-dextrose (CPD), concentrated by plasma removal, and leukoreduced by filtration. The resultant RBC suspensions were divided into equal aliquots and stored in the dark at 4°C in CompoFlex bags (P4159) containing SAGM. Hematocrit and total hemoglobin concentration from RBC suspensions were obtained from quality controls of the blood collection center during the study (hematocrit, 54% ± 1.5%, mean ± 1 SD; hemoglobin concentration, 181.62 ± 6.58 g/L; *n* = 122). Subsequently, RBCs stored for 0, 2, 4, or 6 weeks were allowed to equilibrate at RT for 30 minutes in 15-mL tubes with the caps loosened, before being incubated for 4 or 20 hours at 37°C in a humidified atmosphere of 5% CO₂ in air.

Copper sulfate–ascorbic acid treatment. To generate oxidative stress, 20-mL RBC suspensions were treated on day 1 after blood collection with copper sulfate (0.2 mM) and ascorbic acid (5 mM). Samples were collected for analysis after 1-, 4-, 24-, and 48-hour incubation at 4°C.

CO treatment. To displace oxygen from hemoglobin, RBC suspensions in blood bags were exposed to 100% CO on day 1 after blood collection. A 20-G needle was placed through the septum port, and CO was infused at less than 1 bar, followed by gentle mixing of the sealed bag for 5 minutes. Excess CO in the bag was removed, and the procedure was repeated twice. Conversion of oxyhemoglobin to carboxyhemoglobin was verified spectrophotometrically at 24 hours and 2 weeks after CO treatment, by the increase in absorbance at 541 nm relative to 577 nm (541:577 ratio).

Metabolic supplementation. To stimulate production of NADPH through the PPP, RBC suspensions in blood bags were treated on day 1 after blood collection with a solution containing 20.8 g/L sodium phosphate, 26.8 g/L inosine, 11 g/L sodium pyruvate, and 680 mg/L adenine (PIPA rejuvenation solution, Rejuvesol Red Blood Cell Processing Solution, Zimmer Biomet). Alternatively, blood bags were treated with a solution containing 26.8 g/L inosine only, or 11 g/L sodium pyruvate only, resuspended in SAGM.

The different solutions were added through the septum port at a final concentration of 15% or 30% (vol/vol) on day 0 only or on day 0 and week 3. Samples were collected for analysis after 24 hours, and 2, 4, and 6 weeks of storage at 4°C, followed by 20 hours of incubation at 37°C. Alternatively, PIPA solution was added at 15% (vol/vol) for 1-hour incubation at 37°C, after RBC storage at 4°C for 2, 4, and 6 weeks.

Prx2 dimerization. Dimerization of Prx2 was measured to assess H₂O₂ degradation by Prx2. RBC suspensions in CPD/SAGM were incubated for 15 minutes with 20 mM *N*-ethylmaleimide (NEM), centrifuged at 6,000 *g* for 5 minutes through dibutyl phthalate oil, and lysed in 200 mM NEM in water. Centrifugations were performed at 4°C for samples stored at 4°C and at RT for samples stored at 37°C for 4 or 20 hours. Proteins in the hemolysate were separated on nonreducing NuPAGE gels (Thermo Fisher Scientific) and electrophoretically transferred to nitrocellulose membranes. Prx2 was detected using an anti-Prx2 antibody (R8656, Sigma-Aldrich), anti-rabbit antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories Inc.), and Amersham ECL Detection Reagent (GE Healthcare). Prx2 dimerization was calculated densitometrically and expressed as percentage of Prx2 dimers relative to total Prx2 (dimers and monomers).

Deformability. RBC deformability was measured using an ektacytometer (RheoScan, RheoMeditech Inc.). The elongation index (*EI*) was calculated based on the major axis (*A*) and minor axis (*B*) of the ellipsoid diffraction pattern under a shear stress of 3 Pa, with $EI = (A - B)/(A + B)$ (50).

Osmotic fragility. Resistance of RBCs to osmotic pressure was assessed by diluting RBCs 1:25 in solutions of NaCl with serially decreasing osmolality (ranging from 1 to 9 g/L equivalent to 34–308 mOsm/L). After 30 minutes of incubation at RT, intact RBCs were removed by centrifugation at 1200 *g* for 3 minutes at RT, and an aliquot of the supernatant was added to an equal volume of Drabkin's solution (Sigma-Aldrich). Hemoglobin concentrations were determined spectrophotometrically at 540 nm, and the osmolality yielding 50% hemolysis was calculated.

Hemolysis. In-bag hemolysis was calculated as the ratio between free hemoglobin concentration in the bag and total hemoglobin concentration in RBCs at the initiation of storage. RBC suspensions were centrifuged twice at 2000 *g* for 10 minutes, and the concentration of hemoglobin in the resultant supernatant was determined as described above.

Assessment of H₂O₂ formation. Formation of H₂O₂ in RBCs was assessed by the degree of inhibition of endogenous catalase activity by 1,2,4-AT. This assay is based on the inhibition of catalase due to reaction of AT with catalase compound I, formed when catalase reacts with H₂O₂ (51). Briefly, RBCs were washed twice in PBS, pH 7.4, at 4°C and resuspended at 5% hematocrit in PBS with AT (100 mM). After incubation for 20 hours at 37°C with gentle shaking, RBCs were washed twice in PBS at RT and lysed in water containing β-mercaptoethanol (0.005%), EDTA (2.7 mM), and ethanol (2%); the lysates were then frozen on dry ice. Lysates from 20,000 RBCs were added to 200 μL Tris-HCl 50 mM, pH 8, before H₂O₂ was added to yield a final concentration of 10 mM. Degradation by catalase was monitored as the loss in 230-nm absorbance over 15 minutes in UV-transparent plates (CLS3635, Sigma-Aldrich). The observed AT-dependent decrease in H₂O₂ degradation, expressed as micromoles H₂O₂ consumed per minute per 10⁶ RBCs, was considered to represent H₂O₂ formation (52).

Analysis of NADPH content by enzymatic cycling. Total NADP(H) and NADPH were measured using a colorimetric NADP⁺/NADPH assay kit (65349, Abcam). Hemoglobin and other proteins with MW greater than 30 kDa were removed from RBC lysates using centrifugal filters with a 30-kDa threshold, and filtrates were heated to 60°C for 30 minutes per the manufacturer's protocol to decompose NADP⁺ and permit measurement of the remaining NADPH. Detection reagent was added, and NADP(H) was monitored by absorbance at 450 nm for a period of 3 hours and quantified using standards.

Analysis of NADP(H) content by HPLC-FL-UV. NADPH and NADP⁺ were detected as previously described (53, 54) with slight modifications. Briefly, hemoglobin and other proteins with MW greater than 30 kDa were removed from RBC lysates using centrifugal filters with a 30-kDa threshold (Merck Amicon Ultra-4), and filtrates were frozen and stored on dry ice. Stored samples were thawed on ice and transferred under argon to HPLC vials kept at 4°C, and 100 μL was subjected to HPLC (Agilent 1100 series). NADPH and NADP⁺ were separated on a SUPELCOSIL C18 column (5 μM, 250 × 4.6 mm) with a C18 guard column by gradient elution using mobile phase A (0.1 M K₂HPO₄, pH 6.0) and mobile phase B (20% MeOH in 0.1 M K₂HPO₄, pH 6.0) at 1.3 mL/min. The gradient consisted of 0%–7% mobile phase B (0–6 minutes), 7%–30% mobile phase B (6–11 minutes), 30%–7% mobile phase B (11–16 minutes), and 7%–0% mobile phase B (16–20 minutes). The column was reequilibrated to 100% mobile phase A for 10 minutes. NADPH and NADP⁺ were detected

by fluorescence (Ex = 340 nm, Em = 445 nm) and UV_{254nm}, respectively, and quantified using authentic standards (NADPH, Sigma-Aldrich; and NADP⁺) and ChemStation (Agilent Technologies).

Statistics. Data with $n > 3$ were analyzed using repeated-measures 1-way ANOVA with Dunnett's post hoc test ($\alpha = 0.05$), repeated-measures 2-way ANOVA with Šidák post hoc test (2 conditions), or Tukey's post hoc test (3 conditions) ($\alpha = 0.05$). Data with $n = 3$ were analyzed using Mann-Whitney nonparametric test (1-tailed). Data were analyzed for normality using the d'Agostino-Pearson normality test ($\alpha = 0.05$), following outlier exclusion based on ROUT test (Graphpad Prism Robust regression and Outlier Removal, $Q = 0.01$, $n = 5$ outliers of 432) and found to be normally distributed.

Study approval. The utilization of blood samples from healthy donors for research was approved by the Ethical Committee of the University Hospital of Geneva. Written informed consent was received from participants, and samples were anonymized prior to inclusion in the study.

Author contributions

AR, BAI, MJK, RS, and MS conceived the study, interpreted the results, and wrote the manuscript; AR and JM performed all experimental work; AR performed data and statistical analyses; CS and AA developed and performed analysis of NADPH by HPLC; and NJM and SW contributed to the conception of the study and interpretation of its results.

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