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

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The authors have declared that no conflict of interest exists.

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Abstract

The red blood cell (RBC) storage lesion is a multi-parametric 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 nicotinamide adenine dinucleotide phosphate [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 RBCs oxidative status after restoration of body temperature provides a sensitive tool to detect storage-related alterations, and has the potential to improve the quality of stored RBCs for transfusion.
**Introduction**

Red blood cells (RBCs) prepared for transfusion are kept at 4°C in a blood blank 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 h post-transfusion, as reviewed more than a decade ago,\(^9\) remains unclear. In 2008, Koch et al. studied over 6,000 patients undergoing cardiac surgery, and showed that both short-term and long-term postoperative complications were more common with 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. have introduced the concept of RBC “real age” which may be of greater biological significance than the chronological age of 42 days, which is still accepted by the FDA.\(^13\)

RBC storage-related alterations are commonly studied with cells that have been maintained at 4°C, the standard temperature of RBC storage. However, stored RBCs are most often transfused at room temperature over a period of 4 h 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 post-transfusion 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. In an oxidative environment, molecular oxygen can transform into superoxide radical anion (O$_2^•$), which can then dismutate into hydrogen peroxide (H$_2$O$_2$) through the action of superoxide dismutase (SOD). H$_2$O$_2$ is consumed by several different intracellular biochemical reactions, including those involving catalase, thioredoxin/peroxiredoxin (Trx/Prx) and glutathione/glutathione peroxidase (GSH/GPx) systems.\textsuperscript{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.\textsuperscript{20} Nevertheless, the interconnections among these redox systems, their relationships to RBC functions, and the effects of these systems on survival of transfused RBC 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. 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 Fig. S1A-B). Increased osmotic fragility and decreased deformability were accompanied by progressive hemolysis in the storage bags, with a maximum of 0.8% lyzed erythrocytes after 6 weeks of storage (Supplemental Fig. S1C). 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 h and even more so after 20 h (Fig. 1A-B). Increased osmotic fragility achieved a plateau by 18 h and remained stable with incubation longer than 20 h (Supplemental Fig. S2). Temperature sensitivity was acquired during 4°C storage duration for deformability (week 4 and 6), whereas it was independent of storage duration for osmotic fragility (Fig. 1A-B). In fact, temperature sensitivity was detected in freshly collected samples prior to erythrocyte concentrate preparation (Supplemental Fig. S3). Hemolysis rates were not affected by incubation 20 h at 37°C (Supplemental Fig. S4).

Post-storage incubation of RBCs at 37°C reveals an inability to mitigate oxidative stress
The extent of aminotriazole (AT)-mediated inhibition of catalase activity increased when RBCs were incubated at 37°C compared to 4°C (Fig. 1C), indicating increased formation of H$_2$O$_2$ at elevated temperature. The increased formation of H$_2$O$_2$ at 37°C indicated the importance of antioxidant defenses that protect RBCs during storage. Peroxiredoxin 2 (Prx2), a thiol protein and major H$_2$O$_2$-degrading enzyme in RBCs, showed increased dimerization (a surrogate measure of increased activity in H$_2$O$_2$ degradation) during storage at 4°C, with values mostly less than 15% dimers and wider donor variability after longer storage periods (Supplemental Fig. S1D and S5). Intracellular NADPH remained stable during storage at 4°C. Total NADP(H) increased moderately after 2 weeks but returned to baseline at week 6 (Fig. 1E), with the majority present in the reduced form on week 6 (Fig. 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 h incubation at 37°C led to 60% dimerization of Prx2 (Fig. 1D), and a decrease in total NADP(H) (Fig.1E), with a dramatic loss of NADPH (Fig. 1F). These results were confirmed both by an enzymatic cycling method and by HPLC (Supplemental Figure S6A-B). The observed decrease of NADP(H) at 37°C is consistent with a previous report of NADP(H) modulation upon RBC storage at 25°C (normal room temperature).22

The increased formation of H$_2$O$_2$ 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 for up to 48 h 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$_2$O$_2$, while ascorbate reduces Cu$^{2+}$ back to Cu$^{1+}$. The observed increase in aminotriazole-mediated inactivation of catalase in the presence of copper/ascorbate during 24 h incubation confirms enhanced production of H$_2$O$_2$ (Fig. 2A). Copper/ascorbate treatment of RBCs led to increased Prx2 dimerization within 1 h (Fig. 2B) and a
depletion of total NADP(H) (Fig. 2C) and decreased NADPH/NADP⁺ ratio due to loss of NADPH (Fig. 2D). Increased oxidative stress in RBCs led to subsequent deterioration of RBC functional properties, with a progressive increase in osmotic fragility and hemolysis on a 48 h period (Fig. 2E and 2F). No effect was observed on deformability (data not shown). Thus, the 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. Altogether, these results identify osmotic fragility as a sensitive measure of diminished capacity to mitigate oxidative stress²³⁻²⁵, 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 h. 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 h at 40°C as compared to 20 h at 37°C (Fig. 3A-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 to normal body temperature a febrile state is likely to exacerbate RBC loss in the first 24 h after transfusion.²⁶

**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 $\text{O}_2^-$ and $\text{H}_2\text{O}_2$,\textsuperscript{27} we displaced $\text{O}_2$ 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 (Fig. 4A), indicated by the increase in the 541/577 nm ratio from 0.97 to 1.19, which was maintained at 1.15 after two weeks. CO treatment also led to a decrease in the formation of $\text{H}_2\text{O}_2$ after 20 h at 37°C in freshly processed RBCs, although this decrease was lost after two weeks of storage (Fig. 4B). Conversion of oxyhemoglobin to carboxyhemoglobin completely abrogated Prx2 dimerization, an effect that persisted at 2 weeks (Fig. 4C). Despite prevention of Prx2 dimerization, displacement of hemoglobin-bound oxygen with CO did not prevent increase of RBC osmotic fragility after 20 h at 37°C (Fig. 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 pentose phosphate pathway provides protection of RBC function**

The observed dissociation between Prx2 dimerization and osmotic fragility suggested that redox processes other than hemoglobin autoxidation and $\text{H}_2\text{O}_2$ 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.\textsuperscript{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 a 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 Biomet28,29), that is available for clinical use in the US. 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 (Fig. 5A-B). The PIPA solution also reversed storage-induced osmotic fragility when added for a rejuvenation of 1 h, 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 (Fig. 5C-D). This effect was maintained for 6 weeks when doubling the PIPA to 30% (vol/vol) (Fig. 5D), or when replenishing with fresh PIPA at 15% (vol/vol) after 3 weeks of storage (Fig. 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 (Fig. 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 h at 37°C, as shown both by the enzymatic cycling method and by HPLC (Fig. 5G-H, Supplemental Fig. S6C-D). These results reveal the central role of the PPP in maintaining the redox balance in stored RBCs and indicate that maintenance of NADPH during storage of RBCs has the potential to increase the lifespan 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 of copper-induced osmotic fragility, and a clear decrease in copper-induced hemolysis. These responses were only partial compared to the results obtained with the PIPA solution (Figure 6A-B). Inosine and pyruvate slightly improved storage-induced osmotic fragility for the first two weeks, but to a lesser extent than then 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. RBCs taken directly from 4°C storage may have minor biochemical and cellular alterations that are difficult to associate with physiological relevance for transfused patients. However, these changes underlie more extensive lesions that are revealed upon short-term incubation at 37°C. Part of these revealed changes might be exacerbated in our in vitro experimental system compared to transfused RBCs in vivo, due to 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. NADPH is a major reducing agent in RBCs, that provides reducing equivalents to thioredoxin reductase (TrxR) that are used to regenerate thioredoxin (Trx). Trx, in turn, regenerates Prx2 that degrades H₂O₂. NADPH also protects catalase from inactivation during the metabolism of H₂O₂. While NADPH concentrations were maintained during 6 weeks storage of RBCs at 4°C, suggesting an active PPP, increasing temperature to 37°C decreased the NADPH/NADP⁺ ratio, while substantially increasing H₂O₂ 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 was
observed between the two methods used (Supplemental Fig. S6). One likely explanation of 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.

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 Fig. S7). These results suggest a limited role of 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 ascorbate36 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 deoxy-hemoglobin 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 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.\textsuperscript{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,\textsuperscript{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.\textsuperscript{7,43-45} The increased EMP activity allows for repletion of 2,3-DPG, which promotes hemoglobin-oxygen dissociation in peripheral tissues, and which 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.\textsuperscript{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 two 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\textsuperscript{48} and are consistent with a metabolic study that showed the inability of RBCs to consume adenine after 18 days of storage.\textsuperscript{49} This reveals the complexity of RBC functional lesions, which can not be solely attributed to NADP(H) loss and is likely to require a more complex combination of metabolic enhancers. Preliminary experiments in our hands indicated that inclusion of various amounts of plasma at the time of red cell storage improved osmotic fragility, indicating the need for a multi-dimensional 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.\textsuperscript{25} It is likely that this sub-population of vulnerable RBCs accounts for some of the in vivo hemolysis observed 24 h post-transfusion.\textsuperscript{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 the subset of most fragile RBCs might better be removed 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 (mean age ± 1 SD was 41±15) 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 Fresenius Kabi) containing saline-adenine-glucose-mannitol (SAGM). Hematocrit and total hemoglobin concentration from RBC suspensions were obtained from quality controls of the blood collection center during the study (mean hematocrit ± 1 SD was 54 ± 1.5 %, and 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 room temperature (RT) for 30 min in 15 mL tubes with the caps loosened, before being incubated for 4 or 20 h 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 h incubation at 4°C.

Carbon monoxide treatment
To displace oxygen from hemoglobin, RBC suspensions in blood bags were exposed to 100% carbon monoxide (CO) on day 1 after blood collection. A 20G needle was placed through the septum port and CO was infused at <1 bar, followed by gentle mixing of the sealed bag for 5 min.
Excess CO in the bag was removed, and the procedure repeated twice. Conversion of oxyhemoglobin to carboxyhemoglobin was verified spectrophotometrically at 24 h and 2 weeks after CO treatment, by the decrease in absorbance at 577 nm relative to 541 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 h, and 2, 4 and 6 weeks of storage at 4°C, followed by 20 h of incubation at 37°C. Alternatively, PIPA solution was added at 15% (vol/vol) for 1 h incubation at 37°C, after RBC storage at 4°C for 2, 4, and 6 weeks.

**Peroxiredoxin 2 dimerization**

Dimerization of peroxiredoxin 2 (Prx2) was measured to assess H$_2$O$_2$ degradation by Prx2. RBC suspensions in CPD/SAGM were incubated for 15 min with 20 mM N-ethylmaleimide (NEM), centrifuged at 6,000 g for 5 min 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 h. Proteins in the hemolysate were separated on non-reducing NuPAGE gels (Thermo Fisher), and electrophoretically transferred to nitrocellulose membranes. Peroxiredoxin 2 (Prx2) was detected using an anti-Prx2 antibody (R8656 Sigma), anti-rabbit antibody conjugated
to horseradish peroxidase (Jackson ImmunoResearch) and Amersham ECL Detection Reagent (GE Healthcare). Prx2 dimerization was calculated densitometrically as percentage dimers of total Prx2 (dimer and monomer).

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 1-9 g/L equivalent to 34-308 mOsm/L). After 30 min incubation at RT, intact RBCs were removed by centrifugation at 1,200xg for 3 min at RT, and an aliquot of the supernate added to an equal volume of Drabkin’s solution (Sigma). 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 2,000xg for 10 min and the concentration of hemoglobin in the resultant supernate was determined as described above.

Assessment of H$_2$O$_2$ formation
Formation of \( \text{H}_2\text{O}_2 \) in RBCs was assessed by the degree of inhibition of endogenous catalase activity by 1,2,4-aminotriazole. This assay is based on the inhibition of catalase due to reaction of aminotriazole with catalase compound I, formed when catalase reacts with \( \text{H}_2\text{O}_2 \).\textsuperscript{51} Briefly, RBCs were washed twice in phosphate buffered saline pH 7.4 (PBS) at 4°C and resuspended at 5% hematocrit in PBS with aminotriazole (100 mM). After incubation for 20 h at 37°C with gentle shaking, RBCs were washed twice in PBS at RT, lysed in water containing \( \beta \)-mercaptoethanol (0.005%), EDTA (2.7 mM) and ethanol (2%), and the lysates then frozen on dry ice. Lysates from 20,000 RBCs were added to 200 µL Tris-HCl 50 mM pH 8 before \( \text{H}_2\text{O}_2 \) was added to yield a final concentration of 10 mM. Degradation by catalase was monitored as the loss in 230 nm absorbance over 15 min in UV-transparent plates (Sigma CLS3635). The observed aminotriazole-dependent decrease in \( \text{H}_2\text{O}_2 \) degradation, expressed as µmol \( \text{H}_2\text{O}_2 \) consumed per min per 10\(^6\) RBCs, was considered to represent \( \text{H}_2\text{O}_2 \) formation.\textsuperscript{52}

**Analysis of NADPH content by enzymatic cycling**

Total NADP(H) and NADPH were measured using a colorimetric NADP\(^+\)/NADPH assay kit (Abcam 65349). Hemoglobin and other proteins with MW >30 kDa were removed from RBC lysates using centrifugal filters with a 30 kDa threshold and filtrates were heated to 60°C for 30 min per 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 h and quantified using standards.

**Analysis of NADP(H) content by HPLC-FL-UV**

NADPH and NADP\(^+\) were detected as previously described\textsuperscript{53,54} with slight modifications. Briefly, hemoglobin and other proteins with MW >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, 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 x 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 min), 7-30% mobile phase B (6-11 min), 30-7% mobile phase B (11-16 min), and 7-0% mobile phase B (16-20 min). The column was re-equilibrated to 100% mobile phase A for 10 min. NADPH and NADP+ were detected by fluorescence (Ex = 340 nm, Em=445 nm) and UV254nm, respectively, and quantified using authentic standards (NADPH, Sigma Aldrich, USA; and NADP+), and ChemStation (Agilent Technologies).

Statistics

Data with n > 3 were analyzed using repeated measures one-way ANOVA with Dunnett’s post-test (alpha=0.05), or repeated measure two-way ANOVA with Sidak post-test (2 conditions) or Tukey’s post-test (3 conditions) (alpha = 0.05). Data with n=3 were analyzed using Mann-Whitney non-parametric test (one-tailed). Data were analyzed for normality using the d’Agostini-Pearson normality test (alpha = 0.05), following outlier exclusion based on ROUT test (Q = 0.01, number of outliers n = 5 of 432), and found to be normally distributed.

Study approval

The utilization of blood samples for research is 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

A.R., B.A.I., M.J.K, R.S., and M.S. conceived the study, interpreted the results and wrote the manuscript; A.R. and J.M. performed all experimental work; A.R. performed data and statistical analyses; C.S. and A.A. developed and performed analysis of NADPH by HPLC; and N.J.M. and S.W. contributed to the conception of the study and interpretation of its results.
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Simultaneous extraction and reverse-phase high-performance liquid chromatographic
determination of adenine and pyridine nucleotides in human red blood cells. *Anal
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 h (light grey) or 20 h (dark grey). (C) Hydrogen peroxide formation as described in methods during 20 h at 4°C (white) or 37°C (dark grey) (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 h (light grey) or 20 h (dark grey). (E) Total NADP(H), and (F) NADPH determined by enzymatic cycling at 0, 2, 4 and 6 weeks of storage at 4°C before (white) and after warming to 37°C for 24 h (dark grey) [n=6]. Boxplots show median, 25th and 75th percentile (box) and min/max values (whiskers). * p≤0.05 in repeated measures 2-way ANOVA. (See Supplementary Figure S5).
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 h at 4°C in absence (control) or presence of copper/ascorbate in fresh blood samples [n=6]. (B) Percentage Prx2 dimerization [n=6] in fresh blood samples exposed to copper/ascorbate for 0 (ctrl), 1, 4, 24, or 48 h. (C) Total NADP(H), and (D) NADPH by enzymatic cycling in fresh blood samples exposed to copper/ascorbate for 0 (ctrl), 1, 4, 24, or 48 h at 4°C [n=3]. (E) Osmotic fragility based on solution osmolality leading to 50% hemolysis [n=3], and (F) percentage lyzed erythrocytes [n=3] in fresh blood samples exposed to copper/ascorbate for 0 (ctrl), 4, 24, or 48 h at 4°C. Boxplots show median, 25th and 75th percentile (box) and min/max values (whiskers). Bars show mean value. * p≤0.05 in repeated measures 1-way ANOVA in comparison to control. # p≤0.05 in Mann-Whitney non-parametric test.
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 h (white) or 40°C for 20 h (grey). Boxplots show median, 25th and 75th percentile (box) and min/max values (whiskers). *p≤0.05 in repeated measure two-way ANOVA.
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 (optical density, OD) of untreated (full) or carbon monoxide (CO) treated RBCs analyzed 1 h (dashed) or 2 weeks (dotted) after treatment. (B) Formation of H2O2 by RBC without (♦) or with CO treatment (▼). RBC were stored for 0 or 2 weeks at 4°C, followed by 20 h at 37°C at 5% hematocrit [n=3]. (C) Percentage Prx2 dimerization [n=6], and (D) osmotic fragility based on solution osmolality leading to 50% hemolysis [n=6] in untreated (white) or CO-treated (grey) blood samples. RBCs were stored for 0 or 2 weeks at 4°C, followed by 20 h at 37°C. Boxplots show median, 25th and 75th percentile (box) and min/max values (whiskers). Bars show mean value. * p≤0.05 in repeated measures 2-way ANOVA. # p≤0.05 in Mann-Whitney non-parametric test.
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) percentage lysed erythrocytes [n=3] in untreated RBC samples (●) or samples treated (▼) with 15% PIPA solution (sodium phosphate, inosine, sodium pyruvate, and adenine), followed by copper/ascorbate treatment for 0 (Control), 4, 24, or 48 h 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 grey), (C) samples treated during 1h with 15% PIPA solution after storage (dark grey) [n=3], (D) samples treated with 30% PIPA solution on day 0 (dark grey) [n=6], or (E) samples treated with 15% PIPA solution on day 0 and on week 3 (dark grey) [n=6], and stored at 4°C for 0, 2, 4, or 6 weeks. (F) Percentage lysed erythrocytes [n=6] in untreated RBC samples (white) or samples treated with 15% PIPA solution on day 0 (grey) and stored at 4°C for 0, 2, 4, or 6 weeks followed by 20 h at 37°C [n=6]. Boxplots show median, 25th and 75th percentile (box) and min/max values (whiskers). Bars show mean value. * p≤0.05 in repeated measures 2-way ANOVA. # p≤0.05 in Mann-Whitney non-parametric test.
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) percentage lysed erythrocytes [n=6] in untreated RBC samples (white), samples treated with 15% PIPA solution (light grey), 15% inosine solution (grey), or 15% pyruvate solution (dark grey) followed by copper/ascorbate treatment for 0 (Control) or 48 h at 4°C. (C) Osmotic fragility based on solution osmolality leading to 50% hemolysis in untreated RBC samples (white), samples treated with 15% PIPA solution (light grey), 15% inosine (grey), or 15% pyruvate (dark grey) and stored at 4°C for 0, 2, 4, or 6 weeks [n=6]. Boxplots show median, 25th and 75th percentile (box) and min/max values (whiskers). * p≤0.05 in repeated measures 2-way ANOVA.