

Storage lesion: History and perspectives

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Abstract

Red blood cell concentrates (RBCCs) are the major labile blood component transfused worldwide to rescue severe anemia symptoms. RBCCs are frequently stored in additive solutions at 4 °C for up to 42 d, which induces cellular lesion and alters red blood cell metabolism, protein content, and rheological properties. There exists a hot debate surrounding the impact of storage lesion, with some uncertainty regarding how RBCC age may impact transfusion-related adverse clinical outcomes. Several studies show a tendency for poorer outcomes to occur in patients receiving older blood products; however, no clear significant association has yet been demonstrated. Some age-related RBCC alterations prove reversible, while other changes are irreversible following protein oxidation. It is likely that any irreversible damage affects the blood component quality and thus the transfusion efficiency. The present paper aims to promote a better understanding of the occurrence of red blood cell storage lesion, with particular focus on biochemical changes and microvesiculation, through a discussion of the historical advancement of blood transfusion processes.

Key words: Ageing; Blood cells; Exosomes; Microparticles; Microvesicles; Proteomics; Storage; Transfusion

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Core tip: This review paper puts in perspective the red blood cell storage lesion, which is a hot topic in transfusion medicine. Many different physiological and biochemical pathways are affected by cold storage, and stored red blood cells are clearly very different when compared to freshly drawn erythrocytes. However, most of clinicians are lost in translation because experimental data and clinical data are divergent. Therefore, both fundamental, translational and clinical studies are needed in the near future to provide better care to our patients.

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INTRODUCTION

One major paradox of modern transfusion medicine is related to the fact that donated blood is not transfused directly from donors to patients, but rather undergoes processing for at least 1-2 d. Donated whole blood is most often fractionated into several different components: Red blood cells (RBCs) are packaged as red blood cell concentrates (RBCC) and stored at 4 °C, the platelet component (PC) is stored at room temperature (22 °C) under constant agitation, and therapeutic plasma is generally stored frozen until its use as fresh frozen plasma (FFP). Interestingly, in some emergency rooms, physicians ask for "resuscitation packs" that comprise four RBCCs, four FFP units, and 4-6 PC units. These components are then combined with the aim of recovering whole blood properties.

Decades ago, freshly drawn whole blood was transfused from donor to recipient. The earliest procedures involved blood infusion directly from the donor arm to the recipient arm. Following the development of anticoagulation and short-term preservation techniques, blood could instead be bottled and infused with no necessary contact between the donor and the recipient. Obviously, the reconstituted whole blood presently used in emergency wards is a completely different essential medical product. The modern process offers substantial improvements in terms of quality and immunohematology, and thus of safety. However, it also introduces some caveats that can be underestimated or mistakenly ignored in resuscitation regimens—particularly the possibility of storage lesion.

Here we discuss the various steps that have guided the acquisition of quality and safety over time, along with the supposed minimization of lesion. We start with a retrospective (historical) overview, followed by the natural history of storage lesion. Overall, this look-back aims to provide a better understanding of the clinical impacts of storage lesion in order to prevent unnecessary hazards.

A LOOK BACK AT BLOOD TRANSFUSION PRACTICES

The story of blood transfusion really begins in 1628 with the first description of the human blood flow system by the English physician William Harvey in the "*Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus*". Based on this seminal advancement of human physiology knowledge, blood became viewed as the "river of life". Several physicians theorized that

transference of blood from a healthy person to a sick person could produce curative effects, with targeted sicknesses spanning the spectrum from febrile state to mental disorders.

The injection device first used to attempt blood transfusion was developed in 1656 by the English architect and scientist Christopher Wren, who designed it to test the feasibility of conveying liquid poison into a dog bloodstream. The first blood transfusions are attributed to the British physician Richard Lower, who reportedly performed animal-to-animal and animal-to-human blood transfusions as early as in 1665. The French physician Jean-Baptiste Denis was the first to communicate his experience with animal-to-human blood transfusion, claiming in 1667 to have successfully used a transfusion of sheep blood to cure a 16-year-old boy suffering from fever. However, further attempts were unsuccessful, and one transfusion led to Denis being charged with murder—although this death was ultimately found to have been caused by the victim's spouse and not influenced by the transfusion itself. The controversy led to the 1675 edict of the Châtelet prohibiting blood transfusion in humans and restricting the practice to animal experiments in France.

Approximately one and a half centuries later, in 1829, the English obstetrician James Blundell established the conditions for successful human-to-human blood transfusions. He further documented several successful transfusions and contributed to the development of instruments for blood transfusion. Creite and Landois described the almost systematic lethal agglutination phenomenon observed with animal-to-human transfusion, leading the community to progressively abandon this practice. In the early 1900s, Karl Landsteiner investigated the interactions between blood serum and red corpuscles from different human individuals, leading to his discovery of three groups of blood.

Early blood transfusion was a surgical act involving the dissection of veins or arteries in both the donor and the recipient. End-to-end anastomosis of blood vessels was commonly performed using the Carrel's suture and Crile's cannula methods. Transfusions were thus performed arm-to-arm, meaning that donors had to be present next to the patients. The first direct blood transfusion (arm-to-arm) of World War I (WWI) was performed by the French physician Emile Jeanbrau in 1914. Despite the recommendations made by Hektoen in 1907, the donor and recipient bloods were not cross-matched to ensure their group compatibility, but the transfusion was luckily successful. And so were performed most transfusions during the war. Indeed, the watchword was to accept the risk of blood incompatibility issues rather than let people die from massive hemorrhage.

WWI created a need to dissociate the blood donor from the recipient. Technical development of transfusion devices allowed retrieval of blood from a donor and its injection in the recipient, but such procedures were

often unsuccessful due to blood clotting. In the early 1890s, citrate had been shown to interfere with the clotting process through calcium binding, and Wright^[1] even declared that citrated blood appeared more suitable for transfusion. However, at that time, blood clotting was a minor issue compared with the not yet understood group incompatibility-related agglutination. Moreover, sodium citrate was considered highly toxic and thus unsuitable for human transfusion. In 1914, the Belgian physician Albert Hustin reconsidered the use of sodium citrate to prevent blood coagulation. In 1914-1915, Hustin in Brussels, Agote in Buenos-Aires, and Lewisohn in New York each independently performed successful transfusions of citrated blood to human. This discovery allowed the physical dissociation of recipient from the donor, but it could not be used on a large scale until the end of the war.

Sodium citrate kept blood liquid for up to a few days but not for longer periods, and long-term blood storage was not yet feasible. In 1916, Rous and Turner^[2,3] achieved the first success in erythrocyte conservation, reporting reasonably low hemolysis in rabbit RBCs that were stored in an icebox for four weeks in a solution composed of sodium citrate and glucose. However, such stored RBCs could only be efficiently transfused back to rabbit when the storage duration was 2 wk or less^[3], highlighting the problem of storage-related RBC aging. Thanks to this method, in 1918, Oswald Robertson^[4] reported the efficient storage, transport, and transfusion of 22 "units" of human RBCs stored for 26 d during rush periods at casualty clearing stations. The set-up described by Robertson^[5] can be considered the world's first blood bank. He also developed a specific bottle equipped with a negative or positive pressure pump device for blood collection in citrate solution or infusion of the citrated blood to the recipient^[5].

Due to its duration and the number of involved countries, WWI became a theater of rapid developments and experimentations in blood transfusion. From isolated attempts to rejuvenate life, blood transfusion became part of the routine medical care for soldiers suffering from hemorrhage. After WWI, transfusion-related technical development was limited. Direct and indirect transfusion co-existed. In arm-to-arm transfusion, the control of transfused blood volumes was improved, *e.g.*, by the apparatus of Tzanck (1925) or the pump of DeBakey (1935). The 1930s saw continued use of existing devices for indirect transfusion, *e.g.*, syringes, paraffin-coated tubes, and temporary storage bottles—with the primary improvement being more effective use of sodium citrate. Post-WWI transfusion-related developments mainly focused on the generation of methods applicable in a large-scale practice, and the identification and organization of potential donors for peace times.

The period between WWI and WWII was especially marked by the fact that this emergency procedure entered the field of medicine with extended indications in trauma, surgery, and obstetrics. As blood donation

had become routine practice with soldiers, it began to further penetrate civilian life. The growing extent of blood transfusion practice required more donations and greater organization. Thus, the 1920s witnessed the creation of the first blood transfusion centers, followed by the establishment of blood banks in the late 1930s. In 1936, Spain established the world's first blood bank during its civil war. This was rapidly followed by the appearance of blood banks in the United States of America and in other European countries shortly before WWII.

In 1939, Levine *et al.*^[6] reported the case of a group O patient whose serum induced iso-agglutination (intragroup erythrocyte agglutination) in 80% of tested group O blood samples. One year later, Karl Landsteiner and Alexander Wiener^[7] highlighted the existence of the Rhesus factor, showing that the serum of rabbits immunized with the blood of Rhesus monkeys (*Macaca mulatta*) was reactive to nearly 85% of blood samples from humans (Caucasian), in a manner independent of ABO blood group. Landsteiner *et al.*^[7] further confirmed that this antigen caused the majority of ABO-compatible transfusion reactions. This discovery greatly improved the safety of the blood transfusion process for recipients—apart from the possibility of transmissible infections.

In 1940, the process of fractionating plasma into different protein constituents gained attention. In particular, Edwin Cohn^[8,9] established the basis for plasma fractionation with two papers introducing the separation of equine or bovine serum upon equilibration across membranes using ammonium sulfate or ethanol dilutions with controlled pH, ionic strength, and temperature. The second paper also described a detailed procedure to obtain 50 g of albumin from 2 L of plasma^[9]. Cohn's method allowed the successful production and use of therapeutic lyophilized albumin units during WWII.

Blood preservation saw another substantial improvement in 1943. The alkaline pH of glucose-containing anticoagulant preservative solution led to caramel production during autoclaving, thus requiring separate autoclave heating procedures for the trisodium citrate and glucose solutions, followed by aseptic mixing. To simplify this process, Loutit *et al.*^[10] tried to counterbalance sodium citrate with citric acid. Testing preservative solutions with different sodium citrate/citric acid ratios, they monitored caramel formation and parallel glucose loss, as well as screened blood storage for four weeks at 3 °C-7 °C and examined end-storage hemolysis. Satisfactory solutions were further analyzed for post-transfusion *in vivo* survival and several biochemical parameters—such as spontaneous hemolysis, osmotic fragility, pH, glucose, potassium, and formation of methemoglobin—with comparison to the Rous-Turner and the English Medical Research Council (MRC) preservative solutions in use at the time. They concluded that acidified citrate-glucose (ACD) preservative solutions were satisfactory for blood storage and were recommended for the following

reasons: (1) better survival in recipient circulation; (2) low caramel formation in the autoclaved whole solution; (3) no adverse outcomes during and following transfusion of ACD-stored blood; and (4) an absence of substantial storage-related methemoglobin formation compared with the currently used preservative solutions. ACD blood preservative solutions allowed blood storage for three weeks (based on 24-h post-transfusion survival) and remained in use until the 1957 introduction of phosphate by Gibson^[11], which allowed blood storage for up to four weeks with increased levels of 2,3-DPG^[12].

The 1950s witnessed a critical storage-related technical development. In 1952, Walter *et al.*^[13] presented the utilization of a closed system of plastic bags for blood collection, preservation in ACD, and transfusion. Compared to glass bottles, the plastic bag system had the obvious advantages of reduced bacterial contamination (no contact with air), lighter weight, shock resistance, and ease of storage in refrigerators. In a 1954 publication from the army medical service, Artz *et al.*^[14] reported the use of plastic bags for blood transfusion during the war of Korea. It was determined that blood stored in such bags had lower plasma potassium levels than blood stored in glass bottles. In fact, recent research shows that the plasticizer diethylhexyl phthalate is released from blood bags, and plays a beneficial role in limiting microvesiculation of RBC membranes^[15]. However, in the 1950s, plastic bags were not welcome on battlefields. Gravity-related blood infusion to the recipient was considered inconvenient because “about 30 percent of the blood given in a forward surgical hospital must be given under pressure”, and although plastic bags could be squeezed to force blood out, “it was impractical to assign one corpsman to each bag when 8 or 10 pressure transfusions were being given simultaneously to various patients”^[14].

The next improvements of blood storage involved the introduction of adenine as a constituent of preservative solutions, as proposed in 1962 by Nakao *et al.*^[16]. This research group demonstrated that adenine and inosine enabled ATP regeneration and shape modifications of long-stored RBCs^[17-19], and that ATP level directly impacts RBC *in vivo* viability^[20]. Citrate phosphate dextrose adenine solutions (CPDA-1 and CPDA-2) were licensed for use in the United States during the late 70s/early 80s, although transfusion medicine had already shifted to the use of packed RBC concentrates. The first additive solution used for storage of packed RBC units was sodium-adenine-glucose (SAG)^[21], which was further modified by addition of mannitol (becoming SAGM) to reduce end-storage hemolysis^[22]. Other additive solutions were derived from the original SAG and are currently used worldwide, including anticoagulant solution (AS)-1, AS-3, and AS-5 in the United States and Canada, and MAP in Japan. The guidelines of 75% recipient survival at 24 h post-transfusion and less than 1% (sometime 0.8%) hemolysis allow RBCC storage in SAGM or derivatives

for up to six weeks at 4 °C.

The most recent improvement in blood transfusion involves the removal of leukocytes before or during whole blood processing. Leukoreduction is performed by removal of the buffy coat layer after whole blood centrifugation and/or leukofiltration. This is done because cold-induced leukocyte lysis is believed to damage the stored RBCs. Moreover, leukocyte depletion prevents virus transmission from the donor to the recipient, and inflammatory-like side-effects such as febrile non-hemolytic transfusion reactions and human leukocyte antigens (HLA) immunization^[23]. The ideal duration of RBCC unit storage is debatable^[24].

CURRENT COLLECTION AND STORAGE OF RBC CONCENTRATES

Many efforts have contributed to improving blood transfusion processes, including advancements in procedures for collection, storage, and infusion. Nowadays, blood components are obtained either by processing whole blood donations or by collecting individual components *via* apheresis-driven donation of the required fraction(s). Managing each blood component type separately allows optimized storage of each component in accordance with its intrinsic properties. Moreover, the various labile blood components can be transfused independently of each other, as each can be used to treat different pathologies. Re-transfusion of RBCs can correct anemia secondary to hemorrhage, medullar insufficiency, and anomalies of hemoglobin or erythrocyte membrane synthesis. PCs are transfused in cases of hemorrhagic disorders, and plasma units are used to treat complex hemostatic disorders.

RBCC TRANSFUSION EFFICACY AND CLINICAL OUTCOMES

RBCC efficiency is quite difficult to evaluate because this therapy was introduced before evidence-based medicine was formally established. Transfusion is almost unanimously considered a lifesaving procedure, with many advertisements promoting blood donation stating that “Blood saves lives”. However, this cannot be taken at face value. It must also be considered that, in rare cases, transfusion may harm or even kill the recipient. Moreover, the increasing availability of alternative treatments mean that transfusion is no longer a simple matter of “life or death”, but more often a situation in which transfusion provides a more effective or rapid treatment. Specialists in transfusion medicine tend to argue that transfusion is beneficial, while other physicians cast more doubts on the procedure and express concerns regarding on transfusion-linked hazards. It is important to achieve a realistic view of the benefit-hazard ratio of transfusion processes.

Blood transfusion can be associated with adverse events occurring during the process (transfusion

reaction) or during the 24–48 h following transfusion (post-transfusion hazards). The severity of such events can range from mild to severe and even life threatening. Different transfusion-related adverse events can be classified based on the causative pathogen (immune or inflammatory effector or infectious microbe), the type of reaction (hemolytic or not), and the time from transfusion to event occurrence (immediate vs delayed). Reported serious adverse events include hemolytic transfusion reaction, incorrect blood component transfused, post-transfusion purpura, transfusion-associated circulatory overload, transfusion-associated dyspnea, transfusion-related acute lung injury, transfusion-associated graft-versus-host disease, and transfusion-transmitted infection. Various implemented hemovigilance systems show changed in the incidences of various serious adverse events over time, including an almost complete disappearance of post-transfusion purpura and an important decrease of the reported cases of transfusion-related acute lung injury (with the exclusion of transfusions of plasma from women). Transfusion-related events can also include an increased length of hospitalization, induction of defective post-operative ventilation, deep venous thrombosis, troublesome respiratory syndrome, multiple organ dysfunction syndrome, or even death.

Several studies seem to find that transfusion was more strongly associated with complication risks than with benefits. However, these investigations did not account for several highly important factors, including patient age, severity of the illness requiring blood transfusion, therapeutic regimen, blood group, and the number of blood units being transfused. Thus, the suggestion that blood transfusion carries a great risk may be biased by adverse outcomes that are actually due to a bad health context. Similarly, a greater need for transfusion is associated with a more critical situation and higher morbidity risk. However, other studies have reported no effect of RBCCs storage duration^[25–32], and a few have even found worse adverse outcomes associated with transfusion of fresher blood^[33]. In their 2008 meta-analysis, Marik *et al.*^[34] reviewed the relationship between transfusion and the occurrence of adverse events among critically ill patients. Data from 45 studies (approximately half retrospective and half prospective cohorts) emphasized a link between RBCC transfusion and increased morbidity and mortality in 93% of the cases.

The potential role of blood transfusion in adverse outcomes has also been investigated with regards to the age of the transfused blood products, *i.e.*, the duration of storage before use. In 1993, blood storage duration was first questioned in relation to the biochemical efficacy of transfusion, *e.g.*, oxygen delivery^[35]. In 1997, Purdy *et al.*^[36] performed a retrospective study of septic patients (admission diagnosis) that included the first reported association between blood storage duration and survival. They found that survival did not significantly differ in relation to age, sex, absolute number of trans-

fused RBCCs, or length of the patient's stay at the ICU prior to receiving a transfusion; however, the mean age of the RBCCs transfused to survivors was significantly lower than for non-survivors (17 d vs 25 d of storage in CPDA-1, respectively). Notably, the survivors received a high proportion (85%) of RBCCs stored for less than 10 d, while non-survivors received a high proportion (76%) of RBCCs stored for more than 20 d. Blood groups were not taken into account. In another retrospective study, Vamvakas *et al.*^[37] highlighted an association between wound infection or pneumonia and the storage duration of transfused RBCs in patients undergoing coronary artery bypass graft surgery. Following the introduction of leukoreduction, several authors reported that the process had beneficial effects on known adverse outcomes^[38,39]. Most results impute complications to long-stored RBCs^[40–45]. In 2008, Koch *et al.*^[46] reported that aged RBCCs (more than 14 d of storage) were negatively associated with poor outcomes among pediatric cardiac surgery patients—a finding that had a particularly high impact on the transfusion medicine community. To date, many studies in different countries have examined the use of blood products—with various processing methods and in different pathological cases—to investigate whether long-stored blood components impact transfusion-related adverse clinical outcomes.

The reliability of these results varies based on how the studies were conducted, for example, the number of studied cases; control conditions; data correction with respect to confounding patient-dependent parameters (*e.g.*, age, sex, blood group, illness severity, number of transfused blood units); and study design (*e.g.*, retrospective vs prospective, randomized or not, blinded or not, and dichotomization approach or not). It has been reported that conclusions depend on the number of transfused units^[47]. It appears that many post-transfusion complications were due to immunomodulatory effects attributed to residual leukocytes in RBCCs^[48,49]. Moreover, leukocyte depletion has been recently shown to improve RBC storage in terms of biochemical alterations^[50].

The available literature comprises very differently conducted studies, making it difficult to perform meaningful meta-analyses. One particular issue concerns geography – for example, the processes for blood product preparation differ between America and Western European countries. Van de Watering^[33] has proposed that meta-analyses could be facilitated by more complete reporting of the characteristics of production and storage of RBCs, such as donation type, overnight incubation at room temperature, leukocyte reduction method, preparation of RBC component, and types of bags and additive solution used. Overall, the present data suggest that the duration of blood storage is probably less stringent than initially thought except in specific cases: Namely, extracorporeal circulation and (with less certainty, because there is no suitable comparator) in infants. The European Guide currently recommends giving fresh blood to fetuses, neonates,

Table 1 Overview of the storage lesions

	Lesions	Reversible or irreversible ¹
Biochemical	Loss of metabolic modulation	Reversible
	Accumulation of lactate and pH drop	Reversible
	Ion leakage (K ⁺)	Irreversible
	Decrease of antioxidant defenses	Reversible
	ATP-dependent protein function	Reversible
	Protein oxidation (sulfenic acid)	Reversible
	Protein oxidation (carbonylation)/degradation	Irreversible
	Membrane proteins (band 3 dimerization/accumulation of oxidized proteins)	Irreversible
	Hemolysis	Irreversible
	Lipid oxidation	Irreversible
Morphological	Exposure of senescence markers (phosphatidylserine)	Irreversible
	Shape change	Reversible or irreversible
	Reduced deformability	Irreversible
	Microvesiculation	Irreversible

¹Either *in vivo* or *in vitro*.

and infants, despite the lack of evidence-based studies. This remaining uncertainty urges the transfusion community to address the precise issue of the age of blood. The ongoing ABLE trial will provide some but not all of the needed information.

RBC STORAGE LESION

While the clinical relevance of aged RBCC transfusion on recipient adverse outcomes remains uncertain, there is no doubt that changes at the cellular and molecular levels increase with the storage duration^[51,52] (Table 1). During RBCC storage at cold temperatures (4 °C ± 2 °C), three types of lesions occur at the cellular and intracellular levels: Biochemical lesion, oxidative lesion, and biomechanical lesion.

Erythrocytes undergo biochemical lesion, altering their metabolism. Anaerobic glycolysis occurs in the cytoplasm of RBCs due to the absence of mitochondria. Consequently, energy metabolism through glucose consumption leads to lactate production, which accumulates in the RBC storage solution^[53] and induces its acidification^[54]. This metabolism-related decrease of pH inhibits the glycolysis rate, and affects the 2,3-diphosphoglycerate (2,3-DPG) level through the activities of diphosphoglycerate mutase and phosphatase. After two weeks of storage, RBCs reportedly show 2,3-DPG depletion^[55]. Low 2,3-DPG levels increase the affinity of hemoglobin for oxygen, inhibiting its release and thus altering the oxygenation capacity of stored RBCs. However, 2,3-DPG depletion appears to recover within a few hours post-transfusion.

Cold storage of RBCCs induces inactivation of membrane ionic pumps, as shown by Na⁺ uptake and K⁺ loss throughout the storage period^[55]. Moreover, low-

temperature storage slows metabolic enzyme activities, inducing a progressive decrease of ATP level^[55]. D'Alessandro *et al.*^[56] further reported that accumulation of the metabolic intermediates NADPH and 6-phosphogluconate led to activation of the oxidative phase of the pentose phosphate pathway (PPP). NADPH is required for the reduction of oxidized glutathione (GSSG), recycling reduced glutathione (GSH) to counteract accumulating reactive oxygen species (ROS). However, GSH level has been shown to continuously decrease over the duration of RBC storage^[53]. The literature includes contradictory descriptions of GSSG level, with some studies reporting an increase^[56] and other showing a decrease with no concomitant activation of the PPP^[57].

RBCs also have to deal with oxidative stress due to their constant exposure to oxygen fluctuations. Erythrocytes assist with maintaining circulatory antioxidant levels, particularly by recycling oxidized forms of plasma ascorbic acid^[58], and contributing to the extracellular pool of GSH^[59]. Despite their characteristic absence of mitochondria, RBCs can also act as ROS generators. Indeed, oxyhemoglobin dissociation to deoxyhemoglobin through oxygen release can promote iron electron capture by oxygen. Due to its ferric (Fe³⁺) iron state, methemoglobin cannot bind oxygen, but the enzyme methemoglobin reductase can convert methemoglobin back to Fe²⁺ normal hemoglobin (Hb). This Hb autooxidation occurs at a rate of 3%, and leads to formation of the superoxide radical O₂^{•-}^[60], further producing the highly reactive hydroxyl radical HO[•] through the Fenton reaction^[61]. Such radicals are extremely reactive, possessing a very short half-life of approximately 10⁻⁹ seconds^[62]. Hence, they can non-specifically affect all types of macromolecules within their immediate environment. This can lead to several types of RBC storage lesion, include lipid peroxidation, protein amino acids modifications, and protein backbone breaks^[63,64].

The oxidative lesion is of particular interest, since such damage is not reversed through the transfusion process, in contrast to biochemical modifications. Since erythrocytes are unable to perform protein synthesis, no protein turnover is possible. Damaged proteins accumulate until they are degraded or eliminated from the cell. Redox proteomic studies performed on stored RBCs show increasing hallmarks of oxidative stress throughout the storage period. Several protein carbonylation investigations have indicated that increased storage duration is related to increased carbonylated protein contents associated with the RBC membrane and cytoskeleton^[65-67], or with entire erythrocytes^[53]. Blasi *et al.*^[53] further demonstrated that protein carbonylation was due to increasing oxidative stress concomitant with overactivation of the oxidative phase of the PPP, recruited to counteract ROS. Protein oxidation can also be investigated in terms of cysteine redox status, but such an approach has not yet been employed in the transfusion field. Oxidation reportedly results in

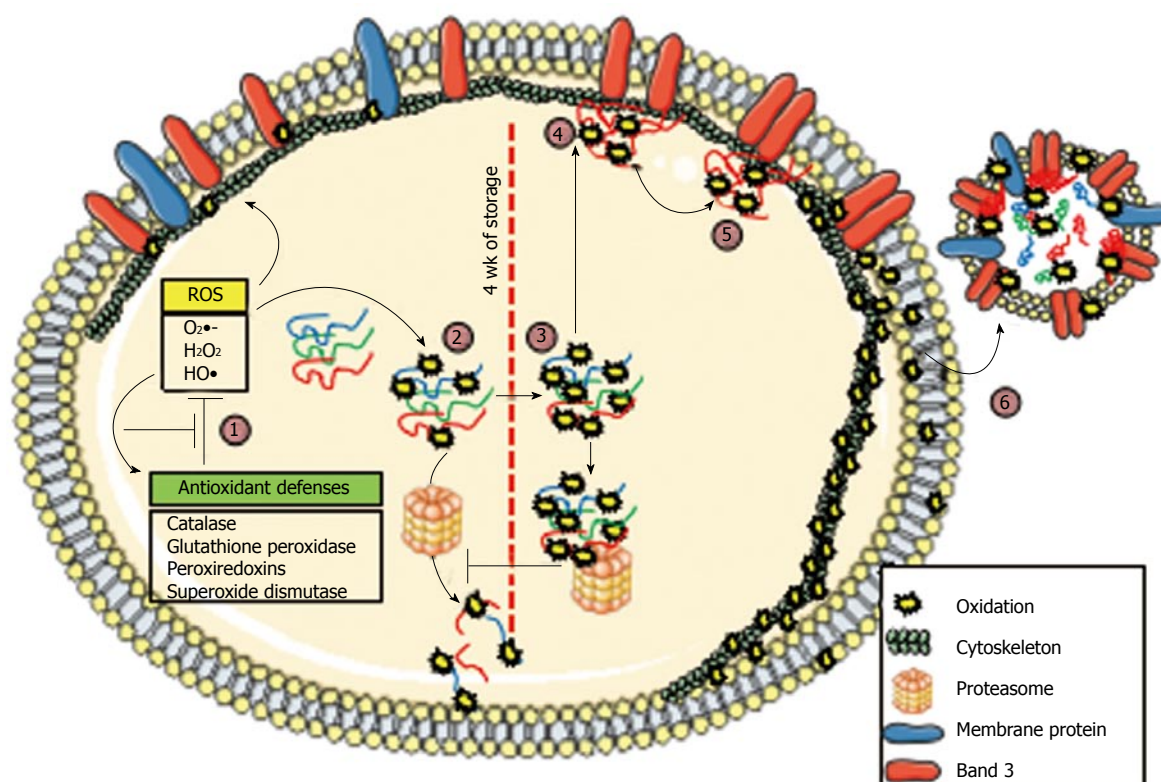


Figure 1 Representation of oxidative pathways involved in red blood cell storage. The model shows an red blood cell (RBC), divided in the middle (dashed line) to show features of early (left half) and late stages (right half) of oxidative damage. Membrane-bound and cytosolic components are indicated in the legend. A microvesicle is shown outside the RBC (right). Progressive oxidation steps are indicated as follows: Step 1: (Left of midline) RBC antioxidant defenses prevent most oxidative damage by reducing reactive oxygen species (ROS), which converts ROS to less-reactive intermediates, or by becoming oxidized by ROS, and then recycling through a restorative mechanism. After prolonged storage, these defenses are overwhelmed by oxidative stress and protein oxidation, which occurs in both the cytosol and the plasma membrane; Step 2: As oxidized proteins unfold, they expose hydrophobic moieties that are recognized by 20S proteasome complexes, which perform proteolysis. Antioxidant defense proteins are also oxidized, and they become susceptible to proteolysis; the lack of defense leads to the overoxidation of RBC content (right of midline); Step 3: At around 4 wk of storage, overoxidized proteins undergo crosslinking, which prevents further degradation; thus, partially-oxidized proteins and damaged proteins accumulate; Step 4: Oxidized hemoglobin aggregates into hemichromes, which bind to the membrane-bound band 3 protein; this binding modifies band 3 conformation, which potentially alters its association with the cytoskeleton, and the hemichromes displace the glycolytic enzymes bound to band 3; Step 5: Hemichrome autooxidation produces ROS, which oxidize cytoskeletal and membrane proteins; Step 6: The release of microvesicles enters an exponential phase, which allows the elimination of RBC aging markers, such as altered band 3 and externalized phosphatidylserine. Reprinted from ref. [65], with permission from Elsevier.

hemoglobin crosslinking, forming hemichromes that covalently bind the cytoplasmic domain of the major integral glycoprotein of the erythrocyte membrane (band 3), inducing its clustering^[68-70]. The induced conformational change creates a neo-antigen site at the erythrocyte surface^[71,72], allowing *in vivo* recognition by naturally occurring auto-antibodies^[73-77]. In turn, this lead to RBC clearance from circulation through complement activation^[78] and phagocytosis by Kupffer liver cells^[79,80]. This is known as the band 3 clustering model.

Hemichromes are also capable of autooxidation, producing ROS in the membrane and cytoskeletal environment. Protein carbonylation, a hallmark of oxidative modifications, reportedly increases in the cytoskeletal protein population during RBC storage^[67]. Such cytoskeletal alteration involves the third type of storage lesion: The biomechanical lesion. Indeed, rheological properties of RBCs apparently undergo alterations throughout the storage period, directly impacting the *in vivo* capacities of transfused RBCs. They become more rigid and thus less able to flow through the capillary

micro-circulation, diminishing their tissue oxygenation power. Most remarkably, their shape is modified, evolving from a normal discoid shape to a spherocyte, with intermediate echinocyte and sphero-echinocyte shapes^[81]. Along with this shape evolution, RBCs release increasing amounts of microvesicles (MVs)^[82], and exhibit reduced deformability^[83], a tendency to aggregate^[81], enhanced adherence to endothelial cells^[84,85], and increasing susceptibility to hemolysis^[86]. The microvesiculation is believed to be a protective mechanism^[87] based on observations of enriched altered band 3 (initiating RBC clearance) in MVs^[24,88-90]. Figure 1 summarizes some of the biochemical events associated with the formation of MVs.

In an editorial recently published in *Blood Transfusion*, Piccin *et al.*^[91] discussed the importance of studying red blood cell microparticles. In the physiological state, phospholipid vesicles of less than 1 μm are present, and their concentrations may vary under pathological conditions. These vesicles generally originate from various types of cells, including red blood cells, platelets,

leukocytes, endothelial cells, and tumor cells. They participate in numerous biological processes (reviewed in^[92-94]). Different types of these vesicles (EVs) have been described, notably exosomes (EXs) and micro-particles (MPs), which are most likely generated *via* different mechanisms, although they share many similarities presenting a challenge regarding their purification and characterization. EXs are small (40 to 100 nm in diameter) spherical vesicles of endocytic origin that are secreted upon fusion of the limiting membrane of multivesicular bodies with the plasma membrane. MVs have been described in blood samples obtained from patients with many different diseases, as well as in cases of storage lesion from red blood cell preparations dedicated for transfusion^[93,95].

Red blood cell-derived microvesicles (REVS) express blood group antigens^[92], and are involved in thrombin generation, and thus in coagulations processes^[96], as well as in inflammation^[97]. Throughout the storage period, RBCs release increasing amounts of MVs^[82], with intense exponential release starting at four weeks of storage. As seen in stored ECs, MVs are also encountered *in vivo* in RBC populations that are heterogeneous regarding the age of cells. It remains unknown whether all cells or only old cells participate in the MV release. Thus, it cannot yet be determined whether cytoskeleton destabilization through hemichrome binding prompts the release of MVs. It is likely that hemichrome binding to membranes^[69] induces conformational changes of band 3, creating a surface neo-epitope^[76] that is recognized by naturally occurring anti-band 3 auto-antibodies^[71,74], favoring *in vivo* RBC clearance. Moreover, storage-released MVs are reportedly enriched in such altered band 3. Thus, it seems likely that hemichrome binding to RBC membranes occurs before the massive release of MVs. One can further hypothesize that hemichrome binding may play a role in signaling towards microvesiculation, perhaps through destabilization of the membrane-to-cytoskeleton association. Overall, microvesiculation appears to be a potential mechanism of defense.

ONGOING RESEARCH TO IMPROVE RBC TRANSFUSION EFFICACY AND SAFETY

In general, the questions regarding transfusion-related adverse outcomes due to aged blood products are not considered a major concern since most RBCCs (notably those of groups A and O) are transfused during the first half of storage, at a mean of 12 d after collection, which is below the 14-d threshold utilized in most published studies and in the European Directorate guide. However, this threshold has not been substantiated by solid and valid scientific studies and with clinical registered trials. Simple dichotomization of fresh vs old blood products has not proven to be helpful for assessing issues related to the duration of RBC storage prior to transfusion, particularly as the authors of such

studies have not agreed on any standard time cut-off for categorizing blood as short-stored or long-stored. If future clinical studies determine such a cut-off time, it may guide changes in all transfusion practices. Future research should be devoted to better evaluating additive solutions, storage conditions, pre-transfusion practices, and even hospital policies (for example, to deliver safer products or patient-specific products, or to limit the needs for transfusion). Several groups are already developing new strategies for better RBC preservation, as well as implementing special storage conditions (*e.g.*, anaerobic storage) and original pre-transfusion practices, such as washing end-stored RBCs.

ADDITIVE SOLUTIONS

RBCC storage represents a substantial improvement in the field of blood transfusion. The development of solutions allowing erythrocyte preservation for up to six weeks has been a long and slow process. The additive solution that are currently used worldwide must be approved by local drug administrations and comply with their guidelines. Almost everywhere, RBCC storage in SAG-M is allowed for 42 d, as such cells are effective at delivering O₂ to patients' tissues and raising Hb levels. However, this is not at all satisfactory in terms of RBC physiology, since RBC can maintain functional capacity for 120 d *in vivo*. Thus, much research has focused on further improving the existing additive solutions, and to otherwise improve the quality of stored RBCCs.

In particular, in the early 2000s, the Hess group published four papers in four years, introducing experimental storage solutions (EAS) allowing effective RBC preservation for 9 wk in EAS-61^[98], 10 wk in EAS-64^[99], 11 wk in EAS-67^[100], and 12 wk in EAS-76^[101]. Their studies reported progressive changes to AS-1-derived EAS that progressively prolonged the RBC storage duration while maintaining a hemolysis rate below 1% and an *in vivo* 24 h recovery of greater than 75%. Compared with the frequently used SAGM and AS-1/3/5 solutions, the EAS was created with a lower salt concentration, higher adenine content (higher AS volume), and a more alkaline AS pH, which seemed to enable longer preservation of ATP and 2,3-DPG concentrations in RBCs. The main drawback of the designed EAS is its alkaline pH that favors dextrose caramelization during the heat sterilization process. Thus, the components must be autoclaved separately then aseptically mixed. Attempts to prolong RBCC storage must account for ease of use, as well as associated variations regarding stability in blood bags, hemolysis, and survival.

REJUVENATION

In addition to developing more efficient additive solutions, the rejuvenation of stored RBCs before transfusion could produce RBCCs of better quality. Rejuvesol™

is a solution comprising pyruvate, inosine, adenine, Na_2HPO_4 , and NaH_2PO_4 . Incubating RBCs in Rejuvesol™ for 1 h at 37 °C, reactivates RBC metabolism allowing replenishment of depleted ATP and 2,3-DPG levels. Afterwards, the rejuvenated cells must be washed to remove excesses of inosine, which are potentially toxic to the recipient. Using this process, Meyer *et al.*^[102] showed efficient increases of ATP and 2,3-DPG in EC samples from RBCs stored in AS-1, AS-3, and AS-5 for 30, 42, 60, 80, 100, and 120 d. Although post-rejuvenation ATP and 2,3-DPG levels were lower in RBCs with longer storage duration, even extremely long-stored RBCs were reportedly capable of a metabolism restart^[103]. Another study conducted by Koshkaryev *et al.*^[104] demonstrated that rejuvenation efficiently reversed the adherence of stored RBCs to endothelial cells, as well as lowered levels of membrane phosphatidyl serine (PS) exposure and intracellular Ca^{2+} and ROS. It is possible that the washing step will induce hemolysis in fragilized older RBCs, but the damaged cells can be selectively removed from the blood product prior to transfusion. Cognasse *et al.*^[105] showed that modulators of inflammation may also be eliminated according. Finally, Pallotta *et al.*^[106] have reported that the addition of antioxidants may prove useful in rejuvenation processes.

PRE-TRANSFUSION WASHING OF LONG-STORED RBCS

Washing of RBCCs has been prescribed by Sir John Dacie for patients suffering from paroxysmal nocturnal hemoglobinuria (Marchiafava-Micheli syndrome). The procedure has been also used in patients with IgA deficiency, and/or in patients presenting with recurrent severe allergic transfusion reactions such as anaphylaxis or severe urticarial reactions not prevented by pre-transfusion antihistamine and corticosteroid administration.

In a recent prospective randomized clinical trial, Cholette *et al.*^[107] investigated the washing of stored RBCs and platelets preceding transfusion to pediatric cardiopulmonary bypass surgery patients. This pre-transfusion practice obviously reduced inflammation markers. Pre-transfusion washing also showed a non-significant association with fewer transfused blood units, and decreased mortality. More recently, Bennett-Guerrero *et al.*^[108] compared different devices for washing long-stored RBCCs. In addition to removing accumulated storage-related compounds (potassium and lactate), they found that the washing procedure induced higher hemolysis and MV release. Free Hb and RBC-derived MVs are known to be scavengers of the vasodilator nitric oxide (NO), and are thus responsible for transfusion-induced impaired vascular function^[109]. However, the washed and recovered RBCs did not seem to be more sensitive to physical stress-induced hemolysis, but rather showed less filtration-related

hemolysis, presumably because older erythrocytes had already lysed during the washing process. With regards to the washing devices, the applied *g* force seemed to impact the quality of the final washed product^[108]. Another focus of interest related to RBC washing is the varying amount of residual plasma in ECs. Depending on the specific processing of the blood product, RBC units can contain anywhere from a few to 100 mL of anticoagulated plasma. Weber *et al.*^[110] demonstrated that in cases where the donor has high-strength anti-HLA class II antibodies, transfusion related acute lung injuries can be attributed to the reactive residual plasma. Although this additional procedure is time-consuming, washing RBCs has the advantage of cleaning the blood products prior to transfusion.

AN OXIDATIVE PATHWAY MODEL OF RBC STORAGE

In addition to storage lesion, oxidative alterations (particularly cysteine residue oxidation) are involved in multiple cellular processes through oxidation-reduction cycles. In the best known example, soluble alcohol oxidase (AO) is less prone to irreversible cysteine oxidation during storage, suggesting that its active site would be preserved, allowing RBCs to fight against ROS. However, by half-way through the RBC storage period, AO shows a higher carbonylation status that correlates with the doubled incidence of oxidation. The global quantitative decrease in soluble carbonylated proteins should be related to preserved AO activity up to the first two weeks of storage. Up to day 26, AO proteins appear to accumulate oxidative injuries through carbonylation. However, from day 26 to day 41, the proportion of this protein family is reduced. This decrease could be explained by the fact that protein oxidation induces recognition and degradation by 20S proteasome complexes. Increasing oxidation of AO defenses, without altering their active sites or making them prone to proteolysis, is likely to change AO enzymes into proteasome inhibitors, as supported by the reported protein oxidation dose-dependent inhibitory effect on P20S activity.

In summary, AO and other proteins accumulate oxidative injuries until around four weeks of RBC storage, making them recognizable by P20S complexes such that they are degraded or they inhibit proteasomal activity. Eventually, AO defenses decrease to the extent that they cannot counterbalance oxidants, allowing overoxidation of proteins that can no longer be taken over by the proteolysis machinery as enzymes from the PPP seem to be. Protein overoxidation induces aggregate formation, and the aggregated forms of Hb are termed hemichromes and bind to band 3 on its cytoplasmic domain. Band 3 proteins are the loci of membrane-to-cytoskeleton binding, and of multiprotein complexes. In particular, band 3 complexes contain proteins belonging to the glycolysis chain. Hemichrome

binding is likely to affect the structure of the cytoplasmic domain of band 3, thus displacing these proteins and potentially destabilizing the cytoskeleton and altering RBC glycolytic functions. Hemichromes like Hb are capable of autooxidation, which may contribute to the oxidative injuries that accumulate on cytoskeleton proteins, membrane proteins, and glycolysis and PPP enzymes. Hemichrome binding and further oxidation of the cytoskeleton and membrane environment could be a signal for high release of aging markers and other modified proteins by microvesiculation.

Several clinical studies on blood age-related transfusion-linked adverse outcomes, as well as research studies on RBCC storage lesion and altered metabolism, suggest that there is a 14-d storage limit after which RBCs are not optimally suitable for transfusion. In contrast, our results suggest that oxidative stress-linked alterations of stored RBCs produce a longer storage limit of four weeks. Transfusion distribution statistics from many hospitals show that half of RBCCs are transfused during the first two weeks of storage, and that around 90% are transfused during the first four weeks. This suggests that, in most cases, transfused RBCs are "healthy" enough to ensure the transfusion goal of increasing tissue oxygenation capacities. However, this conclusion could depend on the storage conditions, particularly the chosen additive solution, and the hospital policies regarding EC delivery. With the advancement of patient-specific medicine, the storage of blood products for transfusion might also eventually become recipient-dependent.

OXYGEN-FREE RBC STORAGE

In frozen and stored RBCs, oxidative stress challenges proteins involved in redox regulation, energy metabolism, and cytoskeleton organization. To deal with this issue, research has investigated the use of anaerobic RBC storage. Yoshida *et al.*^[111] have reported promising results regarding the maintenance of 2,3-DPG and ATP levels during anaerobic RBC storage, as well as diminished MVs release by these RBCs throughout the storage period. In their 2007 study, they applied six gas exchange cycles, achieving a 50-fold reduction of free oxygen concentrations in RBC units^[111]. Though impractical for routine RBCC storage, this setup resulted in increased *in vitro* membrane stability, with reduced hemolysis and MV release rates compared to the conventional six weeks of storage in AS-3. After one week of storage, 2,3-DPG was completely depleted regardless of the oxygenation state. However, ATP preservation was better under anaerobic conditions, with the initial level conserved for up to 7 wk, and only a 15% decrease found after 10 wk, compared to the 64% decrease seen with aerobic storage. The authors attributed this improved ATP maintenance to an increased glycolytic flux with reduced activation of the pentose phosphate pathway due to the lower NADPH oxidation rate.

In a second paper, Yoshida *et al.*^[112] investigated the pH of different anticoagulant solutions, as well as the use of a rejuvenation process, with anaerobically stored RBCs. At any pH, both parameters were better maintained throughout the storage period under anaerobic conditions compared with aerobic storage. Under anaerobic conditions, pH impacted ATP and 2,3-DPG preservation. While the pH of AS showed no effect on hemolysis, they observed a tendency towards reduced microvesiculation under acidic anaerobic conditions^[112]. After seven weeks of acidic anaerobic storage, a rejuvenation process enabled a 24 h recovery of 77.3% of RBCs after 10 wk of storage, with a low rate of hemolysis (0.35%). This 24 h recovery remained acceptable for transfusion after 12 wk of storage, providing that a second rejuvenation process was performed after 11 wk. The use of a novel AS (OFAS-3) produced similarly improved parameters at current maximal storage duration, and the potential to prolong storage to up to 9 wk^[113].

In addition to these markers of RBC storage and *in vivo* recovery data, D'Amici *et al.*^[114] investigated early storage-induced membrane protein degradation and highlighted the role of oxygen depletion in reducing such oxidative damage to proteins. The absence of oxygen can boost glycolysis, but will result in skipping of the pentose phosphate pathway and potentially reduce the GSH defenses.

HOSPITAL POLICIES LEAD TO TRANSFUSION OF FEWER RBCCS

Many countries in Europe and North America are seeing reduced demand for blood transfusion services from hospitals and physicians. Several parameters have contributed to the decrease of the demand: (1) introduction of patient blood management; (2) progress in surgical measures; (3) better anesthesiology; (4) use of cell savers; and (5) implementation of guidelines aiming to clearly describe transfusional indications notably those published by the AABB (<http://www.aabb.org/sa/clinical-practice-guidelines/Pages/default.aspx>), the ISBT (<http://www.isbtweb.org/working-parties/clinical-transfusion/7-red-cell-transfusion-triggers/>) of the American Society of Hematology (www.hematology.org/.../Guidelines-Quality/.../527). However, the number of published guidelines is correlated to the degree of uncertainty of the practices. A Pubmed search (2015-08-21) yielded 559 items using ((guidelines) AND (red blood cell OR erythrocytes) AND transfusion) and with a search performed using (strategies) AND (red blood cell OR erythrocytes) AND transfusion.

The 2013 hemovigilance report from Swissmedic^[115] highlighted a countrywide 6% decrease of RBCC demand. Between 2008 and 2013, this reduction increased to 10.9%. These data are in accordance with new global hospital policies regarding transfusion. For instance, several 2014 papers published in *Transfusion*—the journal launched by the American Association of

Blood Banks (AABB)-described the restrictive blood transfusion practices implemented in hospitals in the United States. A retrospective study from the Roger Williams Hospital of Providence (Rhode Island) also revealed significantly lowered transfusion rates over a 9-year intervention period, with many RBCC transfusions in non-bleeding cases being cancelled or reduced based on patients' Hb levels^[116]. Interestingly, this decrease in RBCC transfusion was closely correlated with a decrease of mortality ($r = 0.88$). Similarly, Yerrabothala *et al.*^[117] from the Dartmouth-Hitchcock Medical Center of Lebanon (New Hampshire) reported a decreased number of transfused ECs in accordance with new local policies, without changes in the lengths of stay or mortality rates. In another example, Goodnough *et al.*^[118] at the Stanford Hospital and Clinics (California) conducted a retrospective study of RBC transfusions and patient outcomes before and after the establishment of a new transfusion policy based on patient Hb level. They reported significant decreases of mortality, length of stay, and 30-d readmission rates thanks to the induced decline of transfusion rates.

In addition to lowered needs for RBCCs, work is being done to artificially produce red blood cells from the culture of hematopoietic stem cells. Luc Douay^[119] has produced some rather promising results in this field, which will most likely benefit patients presenting with complex alloimmunization. Cultured RBCs have proven to be functional regarding deformability, enzyme content, Hb capacity to fix and release molecular oxygen, and expression of blood group antigens. Furthermore, cultured RBCs appeared to survive *in vivo* in humans, exhibiting up to 63% survival at 26 d post-injection. These adaptations are reasonable, and *ex vivo* produced RBCs may eventually become a sustainable surrogate for donated blood. However, artificial blood will not be commercially available soon, and it appears that natural blood will continue to be transfused for a long time.

CONCLUDING REMARKS AND PERSPECTIVES

The impact of the storage duration of transfused blood products, particularly regarding RBCCs, is a currently highly debated issue in the transfusion medicine field. Several clinical studies report a tendency for transfusion of "old blood" to be associated with adverse outcomes in recipients. But the precise definition of "old blood" remains unclear. We cannot yet define a storage duration after which the transfusion of an RBCC would be undoubtedly harmful for the recipient despite suitable transfusion prognostics, *e.g.*, storage hemolysis and *in vivo* 24 h recovery.

There are several obvious reasons why clinical studies cannot delimit any such single storage duration. First, an RBCC is not a globally defined blood component. Variations in many production system and processing

parameters, from blood collection to RBCC delivery, may be quite different among different countries. In particular, the additive solutions used to preserve RBCs during their non-physiological cold storage differ from place to place. Although the formulations of diverse AS seem similar in term of components, some subtle changes appear to substantially impact RBC storage. Energy metabolism is a key factor in RBC maintenance, which appears to be differentially impacted depending on the AS. Another factor complicating the establishment of a storage limit for a guaranteed successful transfusion procedure is the recipient's health status. The same product may have different effects on patients suffering from different healthy conditions. Along the same lines, the same component may have different impacts on two patients with the same hematologic disorder if they are of different ages or different sexes. It is thus difficult to attribute clinical adverse outcomes to a given storage duration, while in fact all other factors influencing a given transfusion procedure are not identical. Most clinical investigations regarding RBCC storage duration focus on one particular clinical situation, with a possible bias because of the topics of interest. In this case, multivariate analyses seems highly desirable to detect evidence-if any exists-that the storage duration of a blood factor has a substantial impact on the ultimate patient outcome, having accounted for a myriad of confounding parameters.

Longer RBCC storage duration leads to greater lesion. Clinical studies have not yet conclusively identified a storage limit before which this lesion will not be hazardous for transfusion recipients. Such a discovery would necessitate a reconsidering of practices regarding blood supply processing and use. Ongoing investigations are exploring new ways to store blood products, and to avoid lesion occurrence. New additive solutions, pre-transfusion procedures, and special storage conditions are being investigated, with encouraging findings. Particularly promising results have been reported with the use of an oxygen-depleted storage environment and the addition of antioxidants to additive solutions. Further research efforts should be invested in such strategies-not necessarily aiming at prolonging storage duration based on erythrocyte stability during storage and after transfusion, but rather at understanding what happens in stored RBCCs. Such efforts could aid in attempts to prevent or correct cell lesion, to find new markers of blood product quality, and most certainly to provide physicians and patients with blood components of the highest quality and metabolic safety.

Finally, research efforts have largely concentrated on blood components. However, from a historical point of view, it is possible that this method was erroneous or less than optimal. We should remain open to the possibility of reintroducing arm-to-arm transfusion, and thus avoiding any lesion attributable to storage, or to otherwise altering the paradigm of transfusion medicine that considers blood components rather than human

blood.

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