

The impact of storage on red cell function in blood transfusion

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Despite the common use of red-blood-cell transfusions in clinical practice, actual beneficial effects of red blood cells have never been demonstrated. On the contrary, several studies suggest that red-blood-cell transfusions are associated with higher risks of morbidity and mortality. The effects of the duration of storage on the efficacy of red blood cells have therefore been questioned in a number of studies. Recent insights into the physiology of red blood cells – such as the role of the hypoxia-induced vasodilator-releasing function of red blood cells – is discussed in relation to the controversy surrounding the use of blood transfusions in clinical practice.

Key words: red blood cell; tissue oxygenation; microcirculation; blood transfusion; transfusion triggers; storage.

HISTORICAL BACKGROUND

The unique function of blood was known by many early civilizations long before the scientific era. It was believed to have a healing ability and to be associated with life, figuring in various beliefs and myths.

The first known transfusion attempt was made, according to legend, in the 15th century, when the blood of three healthy boys was transfused into the veins of the then sick pope Innocentius VIII, unfortunately without success. Two centuries later a Frenchman, Jean-Baptiste Denis, transfused the blood of a calf into a man.

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However, up to the beginning of the 20th century more than a half of the transfused patients died, threatening the development of transfusion medicine. This changed as a result of the findings of Karl Landsteiner who, while investigating failed blood transfusions, identified different blood types, resulting in the ABO and rhesus blood group systems. The development of cross-matching strongly decreased adverse transfusion reactions. A second important development in blood transfusion practice was the introduction by Richard Lewisohn in 1915 of sodium citrate as an anticoagulant storage solution. This important development turned the transfusion of blood into a relatively safe and bearable procedure for both the donor and the patient.

The rapidly evolving transfusion technology solved the problem of short storage time, which became an issue during the Second World War due to the need for large amounts of blood. The development of plastic containers eased the storage and transport of blood units. In the 1950s the separation of blood components, and in the last three decades the developments of additive solutions, rejuvenation and leukodepletion fuelled by the increasing demand for allogeneic red-blood-cell transfusions, significantly improved the quality of stored red blood cells.

RED-BLOOD-CELL PHYSIOLOGY AND ITS ROLE IN OXYGEN DELIVERY TO THE TISSUES

In order to understand the impact of storage on the function of red blood cells it is necessary to review normal red-blood-cell physiology and its role in oxygen delivery to the tissues.

Oxygen delivery to the tissues, in general, is simply calculated as the product of blood flow and arterial oxygen content. This can be described as follows:

$$DO_2 = Q(\text{flow}) \times CaO_2(\text{arterial oxygen content})$$

$$CaO_2 = (\text{Hb} \times SaO_2 \times 1.34) + (\text{PaO}_2 \times 0.003)$$

in which 1.34 represents the oxygen-binding capacity of haemoglobin (mL O₂/g Hb) and 0.003 the solubility coefficient for oxygen in blood (0.003 mL O₂ is dissolved for each mmHg of partial O₂ pressure).

It is obvious from this formula that the decreases in flow, arterial oxygen content (a decrease in red-blood-cell mass or haemoglobin oxygen saturation, or an inability to use the oxygen available in the circulation), and dissolved oxygen should result in tissue hypoxia. It is also obvious that decreases in the variables can be compensated, up to a point, by regulation of other variables.

Pathological conditions such as a decrease in haemoglobin levels during anaemia is tolerated to a certain extent by the action of compensatory mechanisms such as increased blood flow. In addition, a moderate decrease in haematocrit can improve oxygen transport by lowering blood viscosity, thereby improving microvascular perfusion. With this in mind, an optimal haematocrit can be predicted which is lower than the physiological haematocrit. On the other hand, lowering blood viscosity too much, as can happen in haemodilution, can cause a fallout of capillaries and a reduction in functional capillary density.^{1,2}

In healthy adults, tissue oxygenation has a residual capacity. In general, oxygen consumption is approximately one third of oxygen delivery, which allows the body to continue its functions in various conditions, where the changes in DO₂ do not affect VO₂ and tissues do not often encounter hypoxia. Thanks to this residual capacity, a wide range of decreases, causing a decrease in oxygen transport capacity, can be compensated for simply by increases in cardiac output. This was shown by van der

Linden and co-workers, who found that fresh red blood cells were as efficient as blood-flow increases in relieving conditions dependent on oxygen supply.

If the decrease in oxygen-carrying capacity is more than the compensatory mechanisms can handle, further decreases in oxygen delivery (DO_2) can lead to an increase in extraction ratio ($ER = VO_2/DO_2$). Upon reaching a critical point, further reduction in haemoglobin concentration causes oxygen supply to be dependent on VO_2 . Further decreases in DO_2 will result in decreases in VO_2 and leave the tissues hypoxic, and if not corrected this may lead to irreversible tissue damage and organ failure. If such a condition is imminent and decreases in systemic haemoglobin levels occur, the therapy of choice is the administration of blood transfusions.

However, as seen from the viscosity example above, oxygen flux into the tissues and finally into the cells also depends on many other factors, such as blood-flow distribution between organs and within the microcirculation, functional capillary density, red-blood-cell transit times, physical, rheological and functional properties of red blood cells, tissue diffusion coefficient, oxygen transport across the cell membrane, and finally mitochondrial function and oxygen requirement.

The microcirculation has an oxygen-dependent regulatory system which is connected to the systemic circulation, but is also able to regulate and direct blood flow to the tissues depending on the metabolic need of those tissues. The flow of blood in the microcirculation, even under normal conditions, is highly heterogeneous, but by its heterogeneity ensures a homogenous distribution of oxygen in the tissues.³ Therefore, in order to regulate microcirculatory blood flow and thereby oxygen transport to the microcirculation instantly, hypoxia-detecting mechanisms are required. Under normal physiological conditions, this finely regulated system of capillaries, arterioles and venules can supply oxygen in excess of oxygen demand, so that the tissues can continue their function under changing metabolic demands.

THE PHYSIOLOGY OF RED BLOOD CELLS

Besides the negligible amount of oxygen dissolved in plasma, red blood cells are the only cell group responsible for the transport of oxygen to and carbon dioxide from the tissues. In order to fulfil this role, red blood cells use haemoglobin molecules which they produce during their maturation process. The unique ability of haemoglobin to bind tightly to oxygen in the lungs and to release it in the tissues where it is needed stems from the allosteric function of 2,3-diphosphoglycerate (2,3-DPG) produced by the Rapoport–Luebering shunt of the Embden–Myerhof pathway. The significance of 2,3-DPG lies in its ability to lower the affinity of the haemoglobin molecule for oxygen, reflected in a right shift of the haemoglobin–oxygen dissociation curve. This function depends on the amount of oxygen bound to the molecule, the pH of the molecule's environment, and the amount of 2,3-DPG present.

When haemoglobin is fully deoxygenated, the molecule exists in the 'taut' configuration (T state). In this conformation, each of the four haem iron atoms has a low binding affinity for oxygen. When an oxygen atom binds to any one of the four iron atoms in the haem rings, the 2,3-DPG molecule cannot access its binding site, conferring a high oxygen-binding affinity on the remaining haem iron atoms (relaxed or R state).

Red blood cells anaerobically catabolize glucose to lactic acid via the Embden–Myerhof or glycolytic pathway. Since red blood cells do not store glycogen, they must constantly catabolize glucose from the bloodstream via this pathway and the hexose monophosphate shunt in order to obtain energy.

The Embden–Myerhof pathway serves three functions in the red blood cell. The first function is ATP production. Production of ATP is essential for a functioning red blood cell. ATP is the major fuel source of red blood cells, and while several enzymes depend on ATP, the $\text{Na}^+\text{-K}^+$ pump in particular is vital for these cells. The red blood cell's volume is maintained largely by the $\text{Na}^+\text{-K}^+\text{-ATPase}$ in its plasma membrane, which extrudes Na^+ from the cell together with osmotically obligated water molecules. In the absence of ATP, Na^+ is retained and the cell swells. Resultant swollen red blood cells fail to negotiate the microcirculation and are eliminated by macrophages. The second function is the production of 2,3-DPG by an alternative pathway called the Rapoport–Luebering shunt. The third function is NADH production, which is essential for additional critical protection against oxidative damage to the cell from toxic peroxide radicals.

In addition to their oxygen-transporting ability with haemoglobin and the allosteric regulator 2,3-DPG, red blood cells need to be able to travel through a fine network of vessels with diameters $<100\ \mu\text{m}$ where the gas exchange actually takes place: the so-called microcirculation. Normally, erythrocytes have a flexible membrane and can reversibly alter their biconcave, discoid shape, which allows them to pass through capillaries smaller in diameter ($2\text{--}6\ \mu\text{m}$) than red blood cells ($\pm 8\ \mu\text{m}$). To maintain the asymmetric membrane structure, biconcave shape, deformability, surface–volume relationship, intracellular viscosity and other physical properties which allow this flexible structure, red blood cells need energy and hence an adenine nucleotide pool in order to synthesize ATP. The compliant nature of red blood cell membranes (in contrast to the stiffer membranes of leucocytes) is of great importance in this respect, ensuring the successful entrance of the cells into the capillaries (the exchange site), thereby allowing adequate oxygen delivery to the tissues. This property of red blood cells also acts as an in-vivo quality control marker, where stiff old cells are filtered in the spleen and cleared by phagocytes from the circulation.

Besides being a cell without a nucleus and being responsible for oxygen and carbon dioxide transport between organs and lungs, new functions of red blood cells have been found which have led to the idea that red blood cells also play an important role in vascular regulation. Increasing numbers of studies have demonstrated that red blood cells induce vasodilation in the presence of hypoxia and promote oxygen transport. Two major compounds have been proposed in relation to this function: ATP and nitric oxide (NO).^{3–8}

ATP

It has become increasingly clear that, in addition to functioning as an intracellular energy source, ATP can serve as important extracellular signalling molecule. It is now known that red blood cells release ATP in response to hypoxia, pH and mechanical stress.

In mechanical stress, the defects of the spectrin network induced by the deformation of red blood cell⁹ were proposed to play a role in the release of ATP from deformed red blood cells. It is suggested that the partially freed actin at these defect sites may explain the activation of the cystic fibrosis transmembrane protein receptor (CFTR) membrane-bound protein and the subsequent release of ATP by red blood cells subjected to deformations.

In hypoxia, however, Jagger et al¹⁰ suggested that the conformational transitioning of oxygenated haemoglobin (R state) to deoxygenated haemoglobin (T state) due to

oxygen release caused by the decreasing gradient of pO_2 leads to the displacement of phosphofructokinase (PFK) from the cytoplasmic domain of band 3 protein, creating increased glycolysis and ATP accumulation within the red blood cell. Subsequently ATP efflux from the red blood cell is believed to occur via CFTR, allowing ATP to activate endothelial purinergic receptor subtypes, increasing the production of nitric oxide. Extracellular ADP as a product of released ATP, and nitric oxide released from the endothelial cells, are proposed to inhibit further ATP release from the red blood cells. Such a feedback mechanism should protect the organism, since the adenosine concentration inside the red blood cells is almost a 1000-fold higher than that in plasma, and 40% of blood consists of red blood cells.

Nitric oxide

It has been proposed that nitric oxide release during hypoxia is associated with the bioavailability of S-nitrosothiol¹¹ and/or nitrate¹² and nitrite⁷ in red blood cells, both of which are able to donate nitric oxide under hypoxic condition. The first studies regarding nitric oxide focused on the endothelial synthesis of nitric oxide products as a result of ATP release from the red blood cells. However, the recent discovery of a functional endothelial nitric oxide synthase (eNOS) in the red-blood-cell membrane that co-localizes with glycophorin A may also be an important component in this respect.⁸ Such hypoxia-induced, red-blood-cell-associated release of vasodilator substances is now regarded as an important vascular regulatory mechanism, ensuring an oxygen supply adequate for the needs of tissues. However important these vascular control mechanisms may be, other red-blood-cell functions are also important determinants of the ability of red blood cells to deliver oxygen to the tissues.

THE IMPACT OF BLOOD STORAGE

Continued developments in storage techniques have resulted in improved storage times as well as red-blood-cell quality. In this context we refer to 'storage' as liquid preservation, as this is the most common blood preservation technique currently in use. The increasing demand for allogeneic blood transfusions has resulted in millions of liquid-stored allogeneic red blood cell units being used annually for transfusions worldwide. This practice is based on the theoretical expectation that increasing the intravascular mass of red blood cells will increase oxygen delivery to the tissues. However, accumulating evidence is showing that this expectation may not be true, and that there is a negative relationship between the storage time and red-blood-cell viability and function. Additionally, recent findings in observational studies on large populations showed that restrictive transfusion triggers were associated with a better patient outcome. Nevertheless, despite these new findings, and the possibility of using allogeneic blood transfusion alternatives – such as peri/postoperative cell salvage, pre-donation and recombinant erythropoietin administration – liquid-stored allogeneic red blood cells are still the most favoured transfused blood products.

The increasing concerns about the efficacy of allogeneic blood transfusions forces the question about the impact of storage on red-blood-cell function and hence on their use for blood transfusion. First, however, the issue of how the physical and biochemical properties of red blood cells are altered under conditions of storage should be discussed. Indeed, it has been shown that red blood cells undergo a number of changes during liquid storage which affect their viability and their ability to deliver

oxygen to the tissues. We can classify the alterations in two major groups: biomechanical and biochemical.

Biomechanical changes

The first group of changes in red-blood-cell properties is membrane alteration. The structure of the red blood cell is complex, and membrane phospholipids and proteins, cytoskeletal proteins and cytoplasmic components are all related to each other.

Haemorheological alterations – such as red blood cell shape changes, decreased membrane deformability and surface/volume ratio, increased mean cell haemoglobin concentration and osmotic fragility, increased aggregability and intracellular viscosity – can occur during storage and may possibly disturb the flow of red blood cells through the microcirculation and influence red-blood-cell transport of oxygen to the tissues.

During storage, red cells undergo progressive morphological changes, from deformable biconcave disks to echinocytes with protrusions, and finally to spherocytocytes. In parallel with these changes, redistribution and loss of phospholipids in the red-cell membrane by the formation of microvesicles are seen both in storage and in red cell aging, and may contribute to these changes during storage.^{13–16}

The storage-related decrease in red-blood-cell membrane deformability is a crucial change in red-blood-cell properties and is associated with post-transfusional 24-hour survival. The decreased deformability was thought to be associated with reduced ATP levels. While ATP depletion as seen during storage can reproduce many shape changes, a reduction in surface/volume ratio and increases in intracellular viscosity and post-transfusional 24-hour survival of red blood cells precede the decreases in ATP concentration. Only decreases beyond 50% of the ATP concentration can be shown to be associated with decreased mortality, suggesting that the role of ATP depletion in storage-related damage may be limited. Nevertheless, restoring ATP levels in red-blood-cell units appears to correct membrane alterations to a certain level. It is probable that a basal ATP level is necessary for the survival of red cells, and therefore the adenine pool (AMP, ADP, and ATP) has more effect on cellular changes than ATP alone.

Other mechanisms – such as loss or redistribution of membrane phospholipids and protein and lipid oxidations – have been suggested to contribute to the storage-dependent alterations of red-blood-cell membranes. The formation of microvesicles, causing the loss of membrane phospholipids, was identified by Rumsby et al.¹⁶ An alternative mechanism which has been proposed is the internalization of phosphatidylserine (PS) and phosphoethanolamine (PE) from the membrane into the cytosol and loss of asymmetry in the red cell membrane.¹⁷ This suggestion is supported by a recent study by Verhoeven et al.¹⁸ in which the effects of prolonged storage on two different activities affecting the red-blood-cell membrane asymmetry were compared. They studied the effects of storage on flippase, the ATP-dependent aminophospholipid translocase, which moves PS from the outer to the inner leaflet of membrane, compared to phospholipid scrambling which moves PS from the inner to outer leaflet. They demonstrated a decrease in flippase activity starting after 21 days of storage in SAGM (saline, adenine, glucose, mannitol) solution and further decreasing over time. The authors also showed that the correction of storage-induced metabolic changes by increasing intracellular ATP levels only partially restores flippase activity. However, flippase activity could be completely restored when intracellular pH was corrected in parallel with ATP.

In conclusion, the red-blood-cell membrane is certainly adversely affected by storage; however, these alterations appear to be reversible, up to a point, by the use of better storage and rejuvenation solutions. Around one fifth to one fourth of transfused red blood cells are being destroyed within the 24 hours of transfusion. This phenomenon may be explained by changes in red-blood-cell membrane structure triggering immune removal mechanisms, so that these old cells are cleared from the circulation. Band 3 protein, a major membrane protein of the erythrocyte in addition to its suggested involvement in oxygen delivery, is also responsible for triggering the binding of antibodies to antigens and the clearance of these cells from the circulation. Very possibly this mechanism is involved in determining the survival of erythrocytes after transfusion. Other proteins, such as annexin V and CD47, are also proposed to contribute to determination of survival or clearance of red blood cells *in vivo*. While annexin V is a cytosolic protein and is investigated as a sensitive marker for monitoring the potential cellular damage induced by filtration of stored whole blood, CD47 is a cell adhesion molecule, and red blood cells lacking CD47 are believed to be rapidly cleared from circulation by the reticuloendothelial system.^{19–21}

These biomechanical alterations may account for less deformable red blood cells, and may cause even more problems for a microcirculation already under stress under conditions of disease. However, biomechanical alterations are probably not the only problem occurring during storage.

Biochemical changes

2,3-DPG

2,3-DPG is a well-known molecule in red-blood-cell function, as its role in haemoglobin oxygen affinity regulation is crucial for tissues. Therefore, any alteration of 2,3-DPG is believed to be very important, and initial studies on the loss of oxygen-delivering ability of red blood cells during storage were focused mostly on 2,3-DPG. 2,3-DPG is a metabolite and allosteric modifier of haemoglobin and decreases quickly during the first 2 weeks of storage to almost undetectable levels. This decrease leads to an increase in haemoglobin oxygen affinity, which may be an explanation for the decrease of red-blood-cell oxygen-delivering ability during storage. However the 2,3-DPG levels appear to start to recover within several hours, and this may take up to 72 hours after transfusion *in vivo*.²² Considering the fact that blood transfusions are often given to acute patients, waiting for 2–3 days to see the effects of blood transfusion is hardly acceptable.

However, the clinical consequences of completely 2,3-DPG-depleted red-cell units do not seem to be that significant. Theoretically, if 2,3-DPG is not present in red blood cells stored longer than 2 weeks, then approximately two thirds or more of all stored red-cell units would be expected to be 2,3-DPG-depleted. In 2001 d'Almeida and colleagues, investigating the impact of 2,3-DPG depletion in an anaemic oxygen-supply-dependent rat model, compared fresh and old red blood cells stored for 7 days. They were not able to find any differences between the groups, and suggested that 2,3-DPG depletion has a minor physiological impact.²³ Additionally, a recent experimental study showed that although red blood cells were stored for 2–3 weeks and were completely devoid of 2,3-DPG, their oxygen-delivering capacity to the intestinal microcirculation in an oxygen-supply-dependent isovolaemic exchange model did not differ from that of fresh (2–6 days) red blood cells.²⁴ Therefore, we may conclude that decreases in 2,3-DPG levels are reversible and, in the view of storage damage, seem not to be too crucial.

Vasoactive compounds: ATP and NO

An additional biochemical change which occurs in stored red blood cells is the decrease in intracellular ATP levels. ATP, besides playing a secondary role in membrane deformability, is crucial for red-blood-cell function due to its central role in cellular metabolism as an energy source. Sugar transport into the red cell, protective anti-oxidant mechanisms, membrane phospholipid distribution, and all other functions are only possible if ATP is present or can be regenerated in the red blood cell. The newly discovered role of ATP as a vasodilator under hypoxic conditions has highlighted its importance for red-blood-cell function.

The mechanical and hypoxia-induced ATP release is believed to be through a specific membrane-bound receptor, the CFTR.⁹ This function probably depends on a number of factors, including the intracellular adenosine pool, red-cell cytoskeletal and membrane structure, and partially 2,3-DPG presence, in order to detect hypoxia.²⁵ Nevertheless, the complex regulation mechanism of oxygen-sensing and ATP-releasing functions is not very well understood and needs further studies. ATP depletion and the adenine pool in the red cell do not determine the red cell survival directly, but certainly have an important role in red-blood-cell function.

Raat et al²⁴ showed that ATP levels remained unchanged in red blood cells stored for 2–3 weeks, but dropped to 60% in red blood cells stored for 5–6 weeks. This finding was also associated with the oxygen-delivering ability of the red blood cells, and old (5–6 weeks' storage) red blood cells had a reduced oxygen-delivering capacity compared to fresh (2–6 days) and intermediate (2–3 weeks) ones. These findings support the idea that ATP may contribute to oxygen delivery by red blood cells due to its action as a vasodilator and its being released by red blood cells in the presence of hypoxia. This physiological property of ATP may be negatively affected by storage duration.

Another possible mechanism which may account for alterations in the oxygen-transporting capabilities of transfused red blood cells is their ability to generate nitric oxide under acidic and hypoxic conditions. Nitric oxide and its products, besides many other roles in the organisms, can be regarded as being among the major compounds accounting for vascular regulation due to their vasodilatory action on blood vessels. Recent studies have shown that red blood cells are able to release nitric oxide in the presence of hypoxia, and that this nitrite-mediated function accounts for hypoxia-induced vasodilation. An alternative route for hypoxia-induced nitric oxide has been proposed to be the presence of red blood cell-bound S-nitrosothiol.¹¹ The further identification of functional eNOS on red-blood-cell membranes has made the red cell a central player not only in oxygen transport but also in vascular control mechanisms. It could well be that this NO-mediated function of red blood cells may be affected during storage.

In conclusion, the current criteria for the quality of red blood cells for transfusions take biomechanical alterations into consideration as the basis for determining the in-vivo function of the cells. The major properties of blood which are routinely controlled are 0.8–1% haemolysis in stored units, 75% in-vivo survival within 24 hours after transfusions, and volume and haemoglobin content of red blood cells. These are indeed very useful quality parameters; however, biochemical alterations of red-blood-cell properties associated with vascular regulation as discussed above should also be taken into consideration. The alterations which occur during storage appear to be at least partially reversible by use of improved storage conditions, additional solutions, or rejuvenation. An important message in this context is the in-vivo recovery of 2,3-DPG and ATP levels within several hours up to a day after transfusions.

Preclinical and clinical studies

Fitzgerald and co-workers²⁶, using septic oxygen-supply-dependent rats, raised the question of whether the storage duration before transfusion has an impact on tissue oxygenation. The authors compared transfusion with old red blood cells stored in CPDA-1 for 28 days with fresh red blood cells stored for 3 days under oxygen supply conditions. They showed that the transfusion of old cells did not significantly improve the oxygen consumption (VO_2), whereas transfusion of fresh red blood cells acutely increased VO_2 .

Van Bommel et al²⁷, using a rat haemorrhagic shock model, compared the effects of resuscitation with fresh and old red blood cells, the latter stored for 28 days in CPD plasma, SAGM and CPDA-1 solutions. By measuring the intestinal microvascular PO_2 with O_2 -dependent quenching of palladium porphyrin phosphorescence technique, the authors were able to demonstrate that stored red blood cells did not restore the microcirculatory oxygenation, in contrast to fresh red blood cells; however, with the exception of the CPD-stored group, the storage damage was not severe enough to impair intestinal oxygen consumption.

However, d'Almeida et al²³ and Raat et al²⁴ indicate limitations in the previous types of rat models where stored rat red blood cells were used for transfusion. These limitations were the faster aging of rat red blood cells and failure of stored rat red blood cells to regenerate 2,3-DPG, unlike human red blood cells. Raat et al²⁴ developed a rat model able to accommodate human red-blood-cell transfusions. In a randomized controlled study on the ability of fresh (2–6 days), intermediate (2–3 weeks) and old (5–6 weeks) stored human red blood cells to improve gut microcirculatory oxygenation in anaemic oxygen-supply-dependent rats, the authors showed that oxygen delivery capacity was diminished in the old (5–6 weeks) group compared to the fresh and intermediate groups.

In conclusion, the preclinical studies demonstrated the harmful effects of prolonged storage on red-blood-cell functions. However, the results from clinical studies are confusing, and the answer to the question of how important these storage-induced alterations are in vivo, and especially in clinical conditions, remains uncertain.

Marik and Sibbald²⁸ were unable to show any beneficial effects of blood transfusion in septic patients, and Purdy and colleagues²⁹ showed, in severe septic patients, a relation between the age of transfused red blood cells and patient mortality. Keller et al found a relationship between the transfusion of red blood cells older than 14 days and length of hospital stay; however, they did not find a significant increase in length of intensive care stay. Recently Basran and colleagues³⁰ demonstrated that the mean storage duration of the transfused red blood cells was an independent predictor of in-hospital mortality, and associations were found between storage duration and length of hospital and intensive-care stay and acute renal dysfunction.

In contrast, Vamvakas and Carven³¹ could not find any deleterious effects in cardiac surgery patients of transfusion of aged cells. However, in a prospective double-blinded randomized study, Walsh et al³², using red blood cells stored for <5 days versus >20 days, did not observe any significant adverse effects in critically ill anaemic patients. Recently, Hebert et al investigated the effects in cardiac patients of a prolonged storage time of red blood cells used in transfusions. They designed two groups: the standard group received red-blood-cell units with an average storage time of 19 days, while the experimental group received red-blood-cell units with an average storage time of 4 days (5 and 3.4 units per patient, respectively). They found no difference in mortality

and morbidity between the two groups, despite a difference of 15 days in storage time. Van de Watering et al³³ studied 2732 patients who received buffy-coat-depleted red-blood-cell units. They compared patients who received red blood cells stored for longer than 18 days (median 24 days) to patients who received red blood cells stored for less than 18 days (median 13 days). They found no correlation between age of stored blood cells and patient outcome.

Similarly, in large prospective studies on both anaemia and blood transfusion in the critically ill^{34,35}, the storage time of the transfused blood was not associated with a higher mortality or morbidity. However, blood transfusion itself was independently associated with longer intensive-care and hospital stay and mortality.

Based on their findings, the authors above suggested that a limit of 18–28 days be used to identify a red-blood-cell unit as 'old'. If such a threshold were applied in clinical practice, what would the consequences be? To answer this, data on the storage duration of transfused red-blood-cell units are needed. Indeed, in recent large-population studies the storage times of transfused red blood cells were determined. In the Anemia and Blood Transfusion in the Critically Ill (ABC) study³⁴ the mean age of the blood was 16.2 days (± 7 days), whereas in the Current Clinical Practice in the United States (CRIT) study³⁵ the mean age of the blood was 21.2 (± 11.4 days). Interestingly age of blood was found not to be related to any clinical outcome. There was actually a trend, but this did not reach the significance level. However, in both of these studies the number of transfusions was relatively small (12,000 and 4000 respectively) in a total number of 16,000 units. In another study, Raat and his colleagues analysed the age of stored red-blood-cell concentrates in 74,084 units in the Academic Medical Centre in Amsterdam, the Netherlands, between the years 1997 and 2001, for a period of 5 years. They found that the mean storage time was 19.4 ± 7 days, with 37% older than 3 weeks.³⁶

The data above showed that, in a total of 90,000 red-blood-cell units, most of those being used in critically ill patients are 16–21 days old. One third of the patients received transfusions of blood older than 21 days, which supports the idea that dysfunction of these older cells may indeed be a clinical problem. However, while in-vitro studies were able to detect the storage-related changes, most preclinical studies have shown less beneficial, if not deleterious, effects of transfusions of stored red blood cells. One wonders why clinical studies produce such confusing results.

Several hypotheses can be proposed in order to explain this controversy. First of all very few studies have focused primarily on the impact of storage time on red blood cells. Those which did investigate the impact of storage did not monitor tissue oxygenation parameters as ultimate proof of the efficacy of blood transfusions, neither did they measure red-blood-cell properties, instead recording only general patient outcome variables such as mortality and morbidity. The results, therefore, do not allow evaluation of whether transfusion of red blood cells itself, the white-blood-cell burden, or other factors such as severity of disease or risks of transfusion might have caused these results. Under normal physiological conditions only a portion of oxygen delivered to the tissues is actually used. There is a residual capacity for increased demand, so that the tissues can continue to function even under extreme circumstances. Several studies have indeed shown that blood transfusions may increase the oxygen supply to the tissues; however, if oxygen consumption remains stable in patients without critical oxygen delivery status, its benefit would be questionable. Therefore, demonstration of the positive effects of blood transfusions should especially be seen in patients with critical oxygen supply where the compensatory mechanisms cannot handle the alterations in oxygen delivery. If the disease state is not severe in observed patients, this may mask the beneficial effects of blood transfusions.

White-blood-cell burden in stored red blood cells may be another factor affecting transfused blood function, since most studies have been performed prior to the implementation of leukocyte depletion. Several studies have shown the positive effect of leukocyte removal both *in vivo* and *in vitro*. Izbicki et al³⁷ have shown that storage for longer than 3 weeks may play an important role in the development of post-transfusional leukocytosis in transfusion of non-leukodepleted red blood cells by accumulation of interleukin 8. The cytokines and inflammatory mediators are known to be produced by white blood cells during blood storage, and these interfere with immune function. Therefore, theoretically, pre-storage leukoreduction should prevent the accumulation of these products.

Several studies have proposed a difference between the buffy-coat-depleted and leukodepleted red blood cell units.^{37–41} This was explained by the differences in the numbers of leukocytes achieved by these two methods. Buffy-coat-free red blood cells contain about 10^9 leukocytes per litre, whereas this number decreases to 10^6 in leukodepleted red-blood-cell units.

In support of this, Anniss and colleagues⁴² investigated the adherence of red blood cells to vascular endothelium, comparing non-leukodepleted, buffy-coat-poor and leukodepleted red-blood-cell units. They demonstrated significantly less adherence of leukodepleted cells on the first day of storage, and that adherence increased in all groups during 28 days of storage. After 28 days of storage both buffy-coat-poor and leukodepleted red-blood-cell units were less adherent than non-leukodepleted units. This finding supports the idea that white-blood-cell burden, besides causing transfusion-related alterations in immunological response and transfusion-related acute lung injury (TRALI), may worsen the storage-induced changes in the structure and function of the red blood cells.

Underlying diseases may interfere with these results. For instance, in septic patients, increased red cell destruction and shunting may possibly reduce the effects of blood transfusions. For example, Carroll and colleagues⁴³ demonstrated that red blood cells from diabetics had a decreased ATP-releasing ability which is probably associated with an altered antioxidant metabolism.

Another factor may be that the blood chosen to investigate the effects of prolonged storage in some studies may not be old enough, and the use of mixed red-blood-cell units with different storage times in clinical studies may mask the actual impact of storage time. Clinically relevant storage-induced red-blood-cell damage appears to become more obvious in red-blood-cell units which are stored for longer than 3 weeks, so it may be hypothesized that those stored for 18 days may actually be on the borderline. The proportion of these cells in circulation will probably contribute to the clinical efficacy of red blood cells. In clinical practice this can be translated as patients who receive more blood transfusions may be at higher risk, or conversely may benefit more, from this method, depending on how efficient the cells are.

Furthermore, the age of red blood cells at the time of collection may theoretically affect the impact of storage time on red blood cells. In normal physiological circumstances red blood cells have a life-span of approximately 120 days. Therefore a whole blood unit collected from a healthy donor will contain red blood cells with an age spectrum of 0–120 days. It is expected that a proportion of these cells should be older than average or approaching the end of their life span. These cells may undergo more storage-induced changes than younger cells. In support of this, Sparrow et al, in a very recent study, were able to separate young and old red blood cells prior to storage. They suggested a relationship between the age of the red blood cells at the time of blood donation, and changes in the cell-surface expression of cell adhesion molecules

and glycoporin A. Further research should focus on both biomechanical and biochemical alterations in red-blood-cell properties and their clinical importance. Better-designed studies using large populations and in-vivo tissue oxygenation techniques should be performed. The target groups for investigations should especially include patients in a critical oxygen delivery state.

Practice points

- in the absence of adequate evidence, advice to use fresh red blood cells in all patients is unrealistic and unnecessary. Accumulating in-vitro and experimental data, however, strongly suggest the use of fresh red blood cells, especially in critically ill patients who are in an oxygen-supply-dependent state. In such patients, if transfusion is needed, the transfusion of fresh red blood cells may be advised. However, the question regarding relatively old cells still needs to be investigated
- patients' own blood is preferable to stored blood, and therefore the first aim should be to prevent blood loss in patients. Good bleeding control, cell salvage, and avoidance of unnecessary blood sampling for medical reasons may decrease anaemia in most patients. Allogeneic blood transfusion alternatives can be used in some patients
- studies have shown that restrictive blood transfusion triggers may be better for patients. This is supported by most recent guidelines. In practice, if haemoglobin values are >10 g/dL blood transfusions are generally not given, and if <6 g/dL most patients are given at least one blood transfusion. However, decisions on transfusion for patients with a haemoglobin value between these limits should be made individually by the clinicians. The use of haematocrit and systemic haemoglobin values may give a general idea about tissue oxygenation, but they are not the best methods for deciding whether to transfuse because of individual differences between patients in their tolerance of anaemia. Physiological transfusion triggers may be used to evaluate organ function, such as ST elevations observed in electrocardiograms.. However, it should be taken into consideration that each organ has a different residual capacity and may respond differently
- flow redistribution may affect the response to blood transfusions in critically ill patients. Therefore in-vivo monitoring of tissue oxygenation at the bedside is essential. The lack of in-vivo monitoring techniques was a limiting factor in such studies, and can account for why the findings in in-vitro and preclinical studies could not be confirmed in clinical studies. However, these techniques are now available, and some studies have been performed observing microcirculation after blood transfusions. Several patient groups are of particular interest here, such as those undergoing cardiac surgery and extracorporeal circulation, septic patients, haematological diseases and oncology patients. In these diseases, microcirculatory disturbance is caused by different mechanisms, and understanding these changes may improve our understanding of oxygen delivery in critically ill patients
- it appears to be important to revise and standardize the quality criteria for red blood cells by including functional biochemical properties in addition to current regulations based on haemolysis and haemoglobin mass

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