# Cryopreserved packed red blood cells in surgical patients: past, present, and future

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## Abstract

Since the advent of anticoagulation and component storage of human blood products, allogeneic red blood cell transfusion has been one of the most common practices in modern medicine. Efforts to reduce the biochemical effects of storage, collectively known as the red blood cell storage lesion, and prolong the storage duration have led to numerous advancements in erythrocyte storage solutions. Cryopreservation and frozen storage of red blood cells in glycerol have been successfully utilised by many civilian and military institutions worldwide. Through progressive improvements in liquid storage of erythrocytes in novel storage solutions, the logistical need for cryopreserved red blood cells in the civilian setting has diminished. A growing body of current literature is focused on the clinical consequences of packed red blood cell age. Modern cryopreservation techniques show promise as a cost-effective method to ameliorate the negative effect of the red blood cell storage lesion, while meeting the technical and logistical needs of both civilian and military medicine. This review outlines the history of red blood cell cryopreservation, the clinical impact of red cell storage, and highlights the current literature on frozen blood and its impact on modern transfusion.

**Keywords:** glycerol, frozen blood, cryopreservation, thawed blood, military medicine.

## Introduction

The advent of anticoagulant and storage solutions in the early 20th century allowed longer term storage of blood products, enabling the use of allogeneic blood product transfusion to become a vital and widespread therapy in modern medicine. The 2011 National Blood Collection and Utilization Survey estimated that 13.7 million allogeneic whole blood and packed red cell units were transfused in the USA alone during that year, with an additional 65,000 autologous units transfused. Nearly half of all patients admitted to an intensive care unit receive red blood cell transfusions. Large volume transfusion, sometimes termed "massive transfusion"', has been frequently implicated in lung injury since a correlation between the two was first appreciated in the military area in the 1960s[1]. Since then red cell and other blood component therapy has been associated with a multitude of adverse clinical outcomes[2,3]. However, there is no substitute for red blood cell transfusion for the treatment of life-threatening haemorrhage and anaemia in surgical patients. In the USA, 19.8% of red cell units are used by surgical departments, a statistic which increases to 31.2% when including emergency centre, trauma and transplantation needs[4].

Approximately 50,000 adverse transfusion reactions are reported annually in the USA, at an average cost of $ 225.42 per event[4]. These deleterious effects of red cell transfusions have been studied extensively in surgical patients, although causality is difficult to establish in this setting[5]. A meta-analysis of over 20,000 colorectal cancer patients found a correlation between allogeneic red cell transfusions and adverse clinical outcomes including all-cause mortality[6]. Patients undergoing coronary artery bypass grafting who received peri-operative red cell transfusions were shown to have increased early and late mortality[7]. A meta-analysis of 21 studies found a similar association with mortality across many other populations of patients[8].

Furthermore, the duration of storage prior to transfusion has been implicated in worse post-operative outcomes. In a large study of cardiac surgery patients, transfusion of red cells stored for more than 2 weeks was associated with increased post-operative complications (25.9% *vs* 22.4%, p=0.001) and mortality (2.8% *vs* 1.7%, p=0.004)[9]. The cause of this perceived inferiority of older blood is an area of much debate and study. Current hypotheses can be divided into several groups: (i) the depletion of intracellular metabolites, (ii) erythrocyte membrane degradation, (iii) inflammatory mediators, (iv) and haemolysis (Figure 1). These changes taken together are known as the red cell storage lesion. Much frequently reviewed work regarding the mechanism leading to the so called storage lesion already exists, however its practical and clinical relevance is still a matter of debate. A multicentre, randomised study in premature infants was not able to show an effect of red cell storage duration on time spent in intensive care unit or secondary nosocomial infection rate. Randomised controlled multicentre studies are underway to evaluate the effect of the red cell storage lesion in intensive care patients[10] and cardiac surgery patients[11]. Without

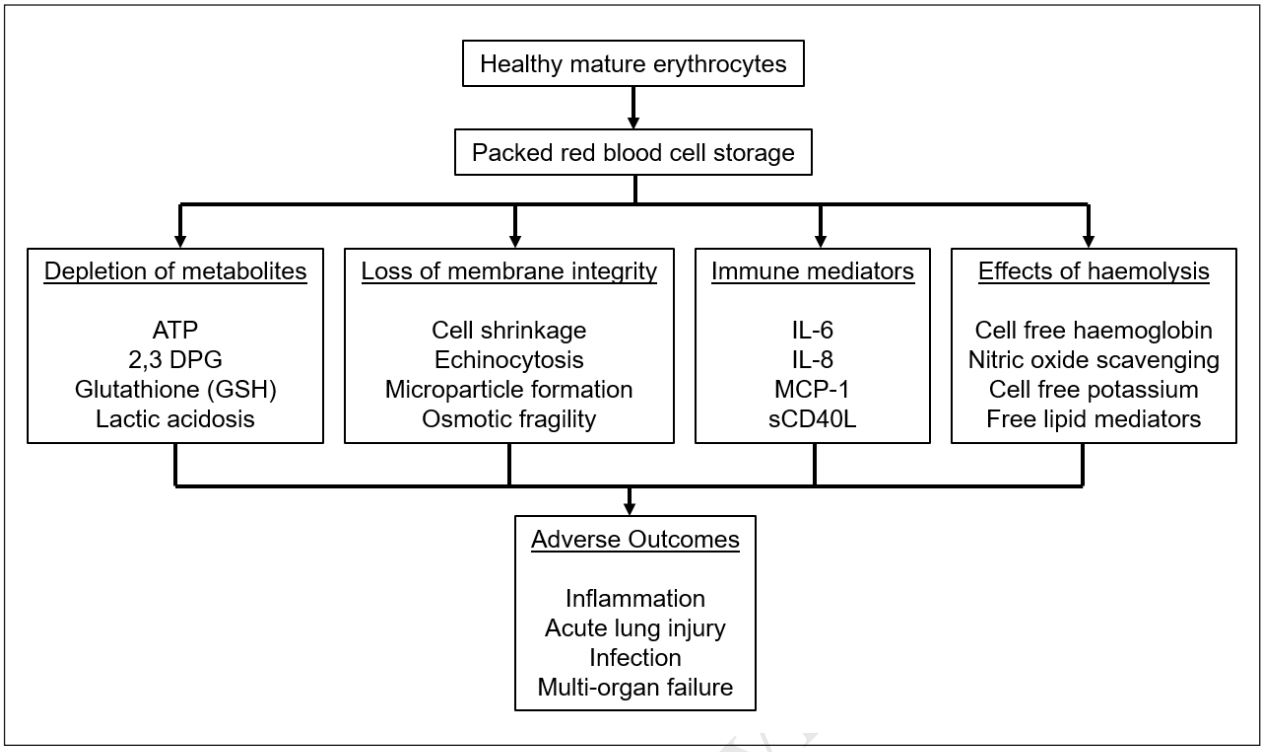


**Figure 1 -** The cumulative red blood cell storage lesion.

conclusive evidence to guide blood utilisation, the overall mean red cell unit storage duration prior to transfusion in the USA is 17.9 days[4].

Recently many investigators have worked to establish more restrictive use of blood products, particularly red blood cells, in the care of diverse populations of patients[12]. The results of the Transfusion Requirements in Critical Care (TRICC) study suggest that restricting the use of red cell transfusion to people with haemoglobin concentrations of 7.0 to 9.0 g/dL does not give inferior outcomes to more liberal strategies[13]. Meta-analyses of randomised trials investigating restrictive transfusion triggers found significant decreases in mortality, cardiac events, bleeding, and bacterial infections[14,15]. Similar analyses of studies in cardiovascular surgical patients, however, found mixed results with restrictive transfusion strategies[16]. Concerns regarding alloimmunisation, antigen exposure in highly sensitised patients, as well as religious opposition to allogeneic blood component therapy make alternative blood product therapy highly desirable. In addition, global military and humanitarian use of blood component therapy is limited by donor availability and bio-vigilance and surveillance of donor blood. Despite these reservations, blood transfusion remains a necessary and life-saving intervention in many surgical settings.

On this background, many investigators have sought to improve the storage of blood products including packed red blood cells. While blood storage at 4 °C is limited by the inevitable development of the red blood cell storage lesion, cryopreservation of packed red blood cells in a frozen state circumvents this problem.

## The science behind cryopreservation of human erythrocytes

Storage of human tissues at temperatures below 0 °C has several advantages when the temperature is sufficiently low to slow or halt the metabolic processes within the cell[17]. Temperatures above 0 °C are not sufficient to maintain viability of most cells for longer than 1 month. Additionally, cooling cells to this temperature may in fact be harmful to the cells due to phase changes in lipid membranes and precipitation of soluble materials. Long term storage of all common cell types requires temperatures below 0 °C but the temperature varies widely across cell types.

The effects of low temperatures on mammalian cells were documented as early as the 19th century. The negligible rate of metabolism at ultralow temperatures promised enormously extended storage of viable tissues; however, irreversible damage to most cells caused by freezing limited its early use. The discovery of the protective effects of glycerol by Polge in 1949 accelerated the development of low-temperature preservation techniques[18].

Many attempts were made to create mathematical models to explain and prevent the damage to cells observed during cooling, but the complexity of the process makes many results difficult to reproduce. Successful cryopreservation protocols are often derived empirically. Initially, the presence of intracellular ice crystals was thought to be the primary determinant of cell damage, although several variables are now known to play significant roles in the viability of tissues after preservation. Because significant osmotic changes and cellular dehydration occur as water is removed from the solution to form ice, the rate of cooling is often critical to the viability of the frozen tissue. Because freezing is an exothermic process, a latent heat of fusion is released as water undergoes a phase change, accelerating the rate of cooling below the freezing point. The rate of freezing has been studied extensively in a variety of cells. In most applications the optimum rate of cooling is near 1 °C per minute. However, many cells, including erythrocytes, maintain high rates of survival after rapid freezing. These observations suggested that cell damage during cooling is multifactorial and involves a combination of cryoprotective solution toxicity and damage during ice crystal formation[19]. Additional observations in the 1960s and 1970s suggested variable haemolysis in erythrocytes was caused by exposure to hyper-osmolarity at different temperatures with significant changes in membrane permeability to cations, sucrose and other solutes[17].

The first successful cryopreservation of human red cells in glycerol was reported by Smith in 1950[20]. The first successful transfusion of human red cells previously frozen in glycerol was reported soon after[21]. During the 1950s, a number of cryoprotective agents were used to successfully freeze and thaw red cells, with glycerol being studied most extensively[22]. Although glycerol is a non-toxic substance, its high intracellular concentration and slow rate of osmosis relative to water makes removal of excess glycerol after thawing necessary to prevent osmotic lysis upon transfusion. The process of glycerol removal can be equally damaging to erythrocytes as the cooling process[23]. In contrast, the warming process, which can be done rapidly, is usually benign. Current practice in the USA has evolved largely from the early work of Valeri and colleagues at the Naval Blood Research Laboratory. First reported in 1966, addition and removal of glycerol using centrifugation resulted in acceptable 24-hour and 7-day post-transfusion red blood cell survival after 3-8 months of storage at −80 °C[24]. Alternative methods of red cell recovery after dilution to remove glycerol include using dialysis[25], Cohn fractionation, serial centrifugation[26,27], and reversible agglomeration[28] techniques. A comparison of these glycerol removal techniques showed satisfactory post-transfusion erythrocyte survival of 85% and minimal haemolysis after as long as 21 years of storage[29]. By this time, however, the widespread availability of commercially available cell washers made these alternatives less attractive[30].

An alternative approach to achieving very cold solid state storage of tissues by very rapid cooling termed vitrification was described in 1984 by Fahy[31]. This technique has been less applicable to preservation of erythrocytes because of practical restraints of cooling large volumes rapidly to its glass transition temperature without ice crystal formation, approximately $3\times10^6$ °C per minute.

The technique developed using red cell suspensions in relatively high glycerol concentrations and freezing at −80 °C became the North American standard due to several pragmatic choices including the early widespread investment in −80 °C mechanical freezers and the high throughput made possible by commercially available and semi-automated cell washers[32]. By the early 1960s, several thousand units had been frozen, thawed and transfused[33]. Early widespread clinical use of these products was inhibited by reports of minor adverse reactions, haemoglobinuria, poor *in vitro* survival and their short post-thaw life-span due to concerns of bacterial contamination[30,34,35]. The subsequent development of more sophisticated post-thaw washing and additive solutions has increased the shelf-life of thawed and deglycerolised red cells, making the product more practical in broad clinical arenas[36-39].

## Past experience with cryopreserved blood products

The first clinical frozen blood programme was established at the United States Naval Hospital in Chelsea (MA, USA). The experience at Chelsea from 1956 to 1960 was published in 1960 by Haynes, who reported normal behaviour and survival of over a 1,000 units of transfused deglycerolised red cells, independently of storage duration and with no significant adverse clinical events[33]. US military experience during the Vietnam War demonstrated that frozen blood products could be used safely even in acutely ill patients[40-42]. Cryopreserved blood still accounted for a very small fraction of the overall number of packed red blood cell units transfused in that conflict[43]. The utilisation of cryopreserved blood was limited due to the labour-intensive and time-consuming processes of thawing and deglycerolisation. The resultant units of packed red blood cells prepared using the Huggin's cytoagglomerator were also limited to use within a few hours of preparation because of concerns regarding bacterial contamination[43].

## Current use of frozen blood

At present, frozen red cells products are approved for use after up to 10 years of storage at −80 °C;

however, current observations show that this is only an administrative limit[44]. The Federal Drug Administration has currently approved products processed in the fully closed ACP 215 Automated Cell Processor (Haemonetics Corp., Baintree, MA, USA) system for up to 14 days after thawing and removal of glycerol. The processes can be performed by a single technician so that a frozen unit can be ready to use within 2 hours. This has the potential to greatly increase the application of frozen red cells in the elective surgical setting.

Previously frozen units of packed red blood cells have been used in military settings for the past 20 years. Since 1993, The Netherlands military has deployed frozen red cells during missions in Bosnia, Afghanistan and Liberia to augment liquid red cells when demands exceeded the available shipments of liquid stored red cells. In the more recent Afghanistan deployment, the ACP 215 was shown to eliminate the need for regular shipments of liquid stored red cells[45]. In their 2006 report, the Dutch military blood bank found that 1,298 out of 1,360 units (95.4%) of cryopreserved packed red blood cells met internal safety criteria 14 days after thawing. The British Army, Scottish National Blood Transfusion Service and American Red Cross have also deployed frozen blood banks internationally[46].

Recent, limited civilian experience with frozen red blood cells in trauma patients found equal safety and efficacy compared to standard-issue packed red blood cells. There is some recent evidence that cryopreserved red blood cells are less inflammatory than liquid preserved red cells. In 2014 Hampton *et al*. reported the results of a prospective randomised study which showed a lower inflammatory response, attenuated fibrinolytic state and increased 2,3-diphosphoglycerate after transfusion of cryopreserved red blood cells compared to liquid preserved red blood cells. Hult *et al*. published a report in 2013 detailing the absent inflammatory effect of transfusion of two units of autologous, cryopreserved red blood cells in healthy volunteers[47-49]. In 2015, Schreiber and his colleagues published the results of a multicentre, prospective, randomised trial which demonstrated that cryopreserved blood is no less safe or efficacious than young and old liquid preserved packed red blood cells in the trauma population[50].

There are many programmes around the globe which store red cells of rare and uncommon blood types at −80 °C. In the USA, the American Red Cross and New York Blood Center have reserves of frozen rare blood types. According to one report, 118 units of frozen rare type blood were transfused after 10 or more years of storage with no evidence of transfusion reactions[51]. Although frozen cells appear to be safe and efficacious, there is still concern when using cells frozen prior to modern nucleic acid testing for human immunodeficiency virus and hepatitis C virus.

## Future directions and reservations about frozen blood products

Nationally, cardiac surgery patients are allocated 10-15% of total available products, the largest proportion in any field of medicine[52]. A recent study found that 48% of a large prospectively enrolled sample of 5,158 cardiac surgery patients received red blood cell transfusions during their initial hospitalisation, with 30% of patients receiving transfusions intra-operatively and a median of 3 units of red blood cells being transfused per patient[53]. This study added to the growing evidence that allogeneic blood transfusion is associated with worse outcomes. Evidence-based recommendations from the Societies of Thoracic Surgeons and Cardiovascular Anesthesiologists report that the need for blood products can be predicted by pre-operative indicators[54]. In situations in which the need for blood transfusion can be reliably predicted, it would be prudent to consider autologous transfusion of pre-donated red blood cells. Cryopreservation of pre-donated autologous blood has the potential to augment the applicability of this practice and minimise the effect of phlebotomy in the pre-operative period[55]. The use of cryopreserved allogeneic red cells represents a potential modality to reduce the effect of the red cell storage lesion.

A study of colorectal cancer patients undergoing curative resection, published by Heiss and colleagues in 1994, found that transfusion of allogeneic blood transfusion was associated with a higher relative risk of cancer recurrence (RR 6.18, p= 0.001)[56]. This association has been replicated in a number of studies and meta-analyses[57,58]. In addition to possible oncological benefits of avoiding allogeneic blood transfusion, the pre-donation of autologous red cells may be of benefit in patients receiving neo-adjuvant therapies.

Human leucocyte antigen allosensitisation due to blood transfusion in pre-kidney transplant patients continues to be a major concern despite adoption of leucoreduction[59]. Previous efforts to improve renal graft outcomes with blood transfusion have largely been replaced by improved immunosuppressive regimens[59,60]. While the total body of literature is mixed regarding the relationship between red cell transfusion, allosensitisation and graft survival, the US Renal Data System 2010 annual report and the United Network for Organ Sharing/Scientific Registry of Transplant Recipients 2010 Annual Report both suggest increases in acute rejection, chronic rejection, antibody-mediated rejection and graft loss with allosensitised recipients. The US Renal Data System report also shows a trend towards longer waiting times associated with

increasing allosensitisation. Taken together there is a trend towards avoidance of pre-transplant allogeneic red cell transfusions. Strategies available for reducing immunological exposure, including single donor and autologous donation, have been limited by red cell storage limits at 4 °C.

The use of cryopreservation to minimise the use of allogeneic blood transfusion is not without its risks. Donation of blood for autologous transfusion places unnecessary risks on the donor if that need is not realised. The additional costs of donation and cryopreservation would be a burden on already strained health care systems. In addition, the once thawed and reconstituted, previously cryopreserved packed red blood cells have a much shorter shelf-life and thus the functional availability of the donated unit is reduced for other patients.

In many instances, the storage and maintenance of allogeneic red cell reserves is not logistically or clinically feasible. Fluctuations in demand and clinical volume are not efficiently paired with increases in donation. The much publicised disposal of over 300,000 units of blood after a large increase in blood donation after the terrorist attacks of September 11th, 2001 highlighted the need for need for more efficient management of this highly perishable and limited resource[43,61]. Furthermore, the total estimated volume of allogeneic blood collected in the USA has declined by 10.6% relative to the population size from 2008 to 2011[4]. The availability of frozen red cells potentially alleviates problems arising from supply and demand mismatches.

The widespread adoption of red cell transfusion took place in the face of massive transfusion as a life-saving procedure. Ethical considerations in the design of clinical studies become more significant as there is accumulating evidence of subtle detrimental effects of red cell transfusion. The observational studies on deleterious effects of red cell transfusion and red cell storage are subject to methodological issues which introduce bias against the current blood allocation system. Indeed, these effects have not been replicated in large, randomised trials. Head to head comparisons between standardly stored packed red blood cells and cryopreserved packed red blood cells in a massive transfusion setting are unavailable.

Despite these difficulties, frozen blood products remain the most promising alternative source of blood products in special situations. As military and expeditionary medicine push surgical care into more remote environments, the advantages of frozen blood products over liquid stored products becomes greater. In these far flung or rural areas, attempts to maintain a constant, reliable supply of liquid stored blood product lead to inevitable waste of outdated blood as well as risks of supply interruptions. As we push into the new frontier of space exploration, a supply of liquid stored blood product will not be available. Frozen blood products offer an attractive alternative blood supply in these unique situations.

While cryopreservation is a promising technique in many special circumstances, logistical and economic hurdles are substantial. The cost of a unit of cryopreserved red blood cells can be three to four times more than that of standard units of packed red blood cells. The cost-effectiveness of the various strategies must be considered in each situation. The other major limitations are the time and resources required to prepare cryopreserved blood for transfusion. Deglycerolisation requires specialised equipment and technical expertise. It can take up to 2 hours to prepare a unit for transfusion. While some specialised centres use multiple blood processors for deglycerolisation which can be operated by a single technician, this level of investment is probably not feasible in most health care systems.

Additional study of the biochemical and metabolic properties of frozen red cell products may reveal even greater advantages compared to current liquid stored red cell products. There are descriptions of apparent reduced antigenicity as well as preserved oxygen-carrying capacity similar to that of liquid stored leucoreduced red cells[62]. As the red cell storage lesion becomes better understood, the replacement of stocks of liquid red cells with on-demand frozen red cell products will become increasingly popular. Since the introduction of the Haemonetics ACP215 the resources (time and manpower) required to produce and use frozen red cell products have decreased substantially compared to those necessary with the numerous manual techniques previously available. With increased utilisation of these products, additional gains in efficiency can be expected. Large-scale manufacture of red cell products may be close on the horizon eliminating reliance on local donation of liquid preserved blood. With long-term stability of red cells at −80 °C, the use of a frozen blood repository for rare blood types can be expanded to the general population, making personalised allogeneic, or even autologous blood donation the new norm.

The assurance of quality remains difficult to quantify. The current standard in the USA for Food and Drug Administration approval requires that 75% of the cells transfused remain in the circulation for 24 hours. Current liquid storage solutions (AS-1, AS-3, AS-5) are essentially equivalent with regard to this 24-hour *in vivo* recovery rate[63,64]. While cryopreserved red cells have been shown to be biochemically similar to those stored using standard preservation techniques, the *in vitro* analysis and post-transfusion survival standards currently in place do not adequately address the relevant

clinical question: does transfusion of liquid or frozen stored red cell products expose recipients to unnecessary risks?

## Conclusions

The modern paradigm guiding red cell transfusions in developed nations reflects decades of slow technological development, regulatory pressures, and a 9.5-billion dollar industry. This has resulted in transfusion practices which are highly variable and lacking an evidence base[5]. While the biochemical and clinical evidence implicating liquid stored red cells in poor clinical outcomes is controversial, frozen red cell products represent a promising adjunct to current blood banking and transfusion practice. More evidence is needed to support the efficacy and safety of these products in the surgical patient.

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