Abstract

Background and Objectives: To review pre-clinical and clinical studies that have evaluated the effects of red cell rejuvenation in vivo and in vitro and to assess the potential risks and benefits from their clinical use.

Materials and Methods: A systematic review and narrative synthesis of the intervention of red cell rejuvenation using a red cell processing solution containing inosine, pyruvate, phosphate and adenine. Outcomes of interest in vitro were changes in red cell characteristics including adenosine triphosphate (ATP), 2,3 diphosphoglycerate (2,3-DPG), deformability, and the accumulation of oxidised lipids and other reactive species in the red cell supernatant. Outcomes in vivo were 24 hour post transfusion survival and the effects on oxygen delivery, organ function, and inflammation in transfused recipients.

Results: The literature search identified 50 studies evaluating rejuvenated red cells. In vitro, rejuvenation restored cellular properties including 2,3-DPG and ATP to levels similar to freshly donated red cells. In experimental models in vivo transfusion of rejuvenated red cells improved oxygen delivery and myocardial, renal and pulmonary function when compared to stored red cells. In humans, in vivo 24 hour survival of rejuvenated red cells exceeded 75%. In clinical studies rejuvenated red cells were found to be safe, with no reported adverse effects. In 1 adult cardiac surgery trial, transfusion of rejuvenated red cells resulted in improved myocardial performance.

Conclusion: Transfusion of rejuvenated red cells reduces organ injury attributable to the red cell storage lesion without adverse effects in experimental studies in vivo. The clinical benefits of this intervention remain uncertain.

Keywords: Allogenic red cell transfusion, storage lesion, rejuvenation
Background and Objectives

Red cell transfusion is one of the most common treatments in hospitalised patients [1]. In 2013, 6.1 million units were transfused in AABB member hospitals in the United States of America, with general medicine being the highest user, and cardiac, general, and orthopaedic surgery combined being the second highest hospital service responsible for the use of red blood cells (RBCs) [2]. However, red cell transfusions have well defined risks. The Serious Hazards of Transfusion (SHOT) scheme estimated that in 2015, the number of SHOT reports received in the UK for red cells was approximately 1.89 million, or, approximately 73% of all SHOT reports for blood components [3]. In addition, observational cohort studies demonstrate strong associations between red cell transfusion and major morbidity including sepsis, lung, myocardial or kidney injury, and death [4]. These associations have been attributed to pathological changes that occur in red cell units during storage, commonly referred to as the ‘storage lesion’ [5]. However, despite strong evidence from experimental models linking the storage lesion to organ injury and immunomodulation in transfusion recipients [5] no randomised clinical trial has shown that these changes are clinically important. In the RECESS trial; a pragmatic multicentre randomised controlled trial (RCT) [6], cardiac surgery patients randomised to receive either younger (stored for <10 days) or older stored red cells (>21 days) had no difference in clinical outcome. The ABLE trial in critical care patients showed similar findings [7]. However, an important limitation of these, and other similar trials is that by 7-10 days of storage red cells already have a significant storage lesion with depletion of 2,3-DPG and nitric oxide buffering, and altered deformability [8]. As a consequence, the contribution of the red cell storage lesion to clinical outcomes remains unclear.

Red cell rejuvenation refers to a process whereby red cells, prior to transfusion, are incubated with a solution containing pyruvate, inosine, phosphate, and adenine (PIPA) at 37°C for 1 hour, and then washed. Rejuvenation acts by restoring glycolytic flux via three processes [9-12]. The first is the conversion of exogenous inosine toward ribose-5-phosphate, which can then be converted to the key glycolytic intermediates, fructose-6-phosphate and glyceraldehyde-3-phosphate. The second is the production of nicotinamide adenine dinucleotide (NAD) via conversion of pyruvate to lactate, which is essential to both inosine metabolism and the generation of 2,3-DPG and ATP via production of 1,3-DPG. The third is the provision of exogenous adenine and inorganic phosphates as substrates for nucleotide synthesis. These processes restore some red
cell characteristics to values comparable to fresh cells. The washing step also removes reactive molecules including cell free haem and oxidised lipids that accumulate in the storage supernatant. Consequently, rejuvenation has been used to extend the shelf life of time-expired red cells by partially reversing the red cell storage lesion. Importantly, the evaluation of rejuvenation processes in clinical trials offers a pragmatic approach to assess the clinical importance of the storage lesion. The aim of the current study was to systematically review the published evidence with respect to the pre-clinical and clinical evaluation (in animal models and humans) of the key cellular and biochemical changes that occur in rejuvenated red cells, and the potential risks and benefits from their clinical use. This work formed part of an application for regulatory approval for a multicentre UK trial of rejuvenated red cells in adult cardiac surgery currently being undertaken by the study authors (NCT03167788) [13].

Materials and Methods

Eligibility criteria

Any experimental or clinical study evaluating the effects of a red cell rejuvenation solution between the 1st of January 1970 and the 16th of January 2017 were considered eligible for this study. Review articles, and studies evaluating the experimental effects of inosine or other components of rejuvenating solutions in isolation were excluded. There were no restrictions on the comparator or the types of outcome measures.

Information sources

Potentially eligible studies were identified by searching PubMed using the following search terms: ((red cell) AND ((rejuvenate) OR (rejuvesol) OR (PIPA) OR (PIGPA) OR (inosine))].

Study selection and data extraction

Two reviewers (G.J.M. and H.A.) identified trials for inclusion independently of each other. Excluded studies and the reasons for exclusion were recorded. The search output was independently screened to identify records of potentially eligible studies, the full texts of which were retrieved and assessed for inclusion. A standardised form was used to extract data from the included studies for review. Extracted information included: year of publication; study objectives; details of the rejuvenation process undertaken; pre-rejuvenation storage solution; details of the rejuvenation intervention
and any comparator; key results. Data extraction forms were completed by one reviewer and checked by a second reviewer.

**Assessment of bias and data analysis**

We anticipated that the studies identified would be too heterogeneous to perform any assessment of bias or to conduct any quantitative analysis. Therefore, a narrative review was performed.

**Results**

Our initial search identified 872 papers (Figure 1). Of these, 820 were excluded on the basis that they did not specifically consider the effects of a red cell rejuvenation solution in stored red cells, or that they were review articles. Details of the analyses were not accessible in 2 studies leaving 50 papers for the final review (Tables 1-5). We grouped together 41 studies that evaluated the effects of rejuvenation in vitro, and on 24-hour post-transfusion survival in vivo, as these were often reported together. This group was then sub-divided into studies that considered rejuvenation only (n=19), rejuvenation-freeze-thawing-deglycerolisation (n=13), and rejuvenation-washing without glycerolisation (n=9). We identified a further 6 studies that had primarily considered the effectiveness of rejuvenated red cells in experimental animal models in vivo, and 3 studies where the sole purpose of the study was to evaluate the effects of rejuvenated red cells in human clinical studies. Data from an experimental study from the authors’ own institutions was also included.

**Figure 1. Overview of search**

**Studies evaluating the effects of rejuvenation in vitro, and on 24-hour post-transfusion survival in vivo**

**Rejuvenation only (Table 1)**

Early studies in the 1970s considered the effects of a rejuvenation solution on red cells stored in Acid Citrate Dextrose (ACD). These confirmed that co-incubation with a solution containing pyruvate, inosine, glucose, phosphate and adenine (PIGPA) restored ATP and 2,3-DPG levels to those of fresh red cells. These levels were maintained for several days following rejuvenation [14]. A similar study also demonstrated restoration of membrane properties and normal red cell morphology [15]. The high glucose concentration in the PIGPA solution led to caramelisation at room
temperature and this was omitted to form the PIPA solution that would later be branded as rejuvesol® Red Cell Processing Solution (rejuvesol Solution) (Table 6). Rejuvesol Solution was shown to restore 2,3-DPG, ATP, morphology, p50, and deformability in RBCs after storage in more modern additives including Additive Solution 1 (AS-1), Additive Solution 3 (AS-3), Additive Solution 5 (AS-5) and saline, adenine, glucose, mannitol (SAGM) [16-20]. Subsequent studies also considered other effects of rejuvenation including reduced adherence to vascular endothelial cells under flow (AS-1) [21] and reduction in haemolysis [22].

More recent studies have sought to examine the limits of RBC rejuvenation. Meyer and colleagues demonstrated that the potential for rejuvenation diminished after 30 days of storage in AS-1, AS-3 & AS-5 [11]. Tchir and colleagues demonstrated that reversal of storage related changes in deformability and morphology were not evident after 28 days, although ATP and 2,3-DPG were restored [22]. Barshtein and colleagues also documented that rejuvenation of red cells initially stored in citrate, phosphate, dextrose and adenine (CPDA-1) for 5 weeks did not restore mechanical fragility and deformability to that of fresh red cells [23]. In an early study, the failure of rejuvenation to reverse morphological changes in spheroechinocytes was attributable to reductions in membrane volume post shedding of microvesicles with a resulting inability to restore the surface area to volume ratios of normal discocytes [24]. Bayer and colleagues have also reported that rejuvenation of red cells stored in SAGM for 42 days does not reverse the dysfunction of anti-oxidant mechanisms related to storage [25]. There are conflicting reports on the effects of rejuvenation on the activity of deoxygenated haemoglobin as a metabolic switch that shuttles glucose from the pentose phosphate pathway (PPP) and Embden Meyerhof pathway (glycolysis). In healthy red cells, haemoglobin deoxygenation results in the de-coupling of glycolytic enzymes from Band 3 proteins leading to increased glycolysis and ATP synthesis and export. Extracellular ATP promotes vasodilatation and this is thought to contribute to the autoregulation of microvascular oxygen supply and demand. After prolonged storage and ATP depletion this oxygen dependent switch in red cell metabolism is not observed. This leads to PPP pathway activation and the synthesis of nicotinamide adenine dinucleotide phosphate (NADPH), an anti-oxidant [10]. Messana and colleagues demonstrated that rejuvenation did not restore this metabolic modulation after 21 days of storage, and in fact increased glucose conveyance to the PPP despite restoring 2,3-DPG levels [10]. Conversely, Kirby and colleagues demonstrated that rejuvenation restored ATP synthesis and export
in red cells stored in AS-3 for >35 days, and reduced cell adhesion to murine vascular endothelium in vivo [9].

Although most studies of rejuvenation have been at 37°C, others have demonstrated the benefits of rejuvenation during anaerobic storage at 4°C [26]. The combination of cold rejuvenation and anaerobic storage results in supraphysiological ATP levels (mean 7 μmol/g Hb), sub-normal 2, 3-DPG levels (mean 10 μmol/g Hb). This combination also results in acceptable 24 hour red cell recovery; 77% [26].

**Rejuvenation-freeze-thaw-deglycerolisation (Table 2)**

Early studies evaluated the effects of PIGPA rejuvenation of citrate, phosphate, dextrose (CPD) stored red cells, followed by glycerolisation, freezing, thawing (after a period of storage), and deglycerolisation. These cells were then stored for up to 72 hours in a salt buffer solution (sodium chloride and glucose, or sodium chloride, glucose, and phosphate) [27, 28]. *In vivo* studies demonstrated excellent red cell recovery and 24 hour survival rates; 90% and 75% respectively after 1.5 years of storage [29], and similar rates even after 3 years of storage [30]. Further studies demonstrated that this technique could be refined with improved collection bag design and processes for glycerolisation, thawing and deglycerolisation [27, 28, 31], could be applied to small transfusion volumes [32], and had theoretical benefits in extracorporeal circuits due to the impact on reducing haemolysis [33]. In studies of the effect of transfusion on myocardial function, perfusion by rejuvenated human red cells frozen for up to 6 months, followed by thawing and deglycerolisation, was found to improve myocardial oxygen consumption and reduce markers of cellular hypoxia in isolated fibrillating dog hearts when compared to perfusion with non-rejuvenated cells [29].

More recent studies have documented similar effects of rejuvenation-freeze thaw deglycerolisation in red cells stored in modern additive solutions such as AS-1, AS-3, and AS-5 [19]. Rejuvenation results in supra-normal 2,3-DPG and ATP levels following storage in modern additive solutions, and comparable freeze thaw deglycerolisation, with recovery at 85%, and 24 hour *in vivo* survival at 75-85% [18, 34]. Most recently Alessandro and colleagues have demonstrated that rejuvenation-freeze-thaw deglycerolisation in 42 day old AS-1 stored red cells upregulated glycolysis, increased levels of PPP intermediates and partially rescued glutathione biosynthesis when compared to 42 day cells. Evaluation of lysophospholipids also suggested activation of recycling pathways of damaged membrane lipids by rejuvenation [35].
Rejuvenation-washing without glycerolisation (Table 3)

The possible infusion of residual inosine during transfusion of rejuvenated RBC was identified as having the potential to cause an adverse effect due to its association with uric acid synthesis and gout. Studies that sought to evaluate rejuvenation as a clinical intervention therefore washed red cells post rejuvenation and prior to transfusion, using commercially available mechanical cell washers. The re-suspension solution in early experiments was 0.9% sodium chloride solution, with the later addition of glucose and ultimately phosphate to allow post rejuvenation storage for longer periods [36]. Rejuvenation-washing recovery in vitro was 98% in these experiments, where red cells were stored for 24 hour and 72 hours post wash, with in vivo 24 hour post transfusion survivals of 82% and 79% respectively [36].

Studies evaluating the effects of rejuvenated red cells in experimental in vivo animal models (Table 4)

Rodents

Raat and colleagues compared the effects of exchange transfusion of SAGM human red cells stored for 3 days versus rejuvenated or non-rejuvenated human red cells previously stored for up to 6 weeks in a rat model of isovolaemic exchange transfusion [37]. Red cells were rejuvenated using rejuvesol Solution, according to the manufacturer’s instructions, resulting in the reversal of storage related reductions in 2,3-DPG and ATP. In study subjects, isovolaemic haemodilution resulted in renal hypoxia. This was reversed by the transfusion of fresh 3 day stored red cells and by rejuvenated 6 week stored red cells, but not by the transfusion of non-rejuvenated 6 week stored red cells. The effects of transfusion on oxygenation were attributed in part to increases in renal blood flow with fresh and rejuvenated cells. Gelderman and Vostal evaluated post-transfusion in vivo red cell recovery in an immunodeficient mouse model and suggested that the rejuvenation process improved roller pump-induced physical and osmotic stress resistance of stored RBCs [38].

Pigs

Wozniak, Cardigan and colleagues evaluated the effects of red cell rejuvenation on transfusion associated organ injury in an established swine model of transfusion associated organ injury [39]. In this model, 14 day pig red cells exhibited characteristics comparable to day 35 human red cells. Transfusion of 14 day stored pig red cells resulted in acute lung injury as a result of platelet and leucocyte activation. In addition,
transfusion of 14 day stored red cells resulted in endothelial activation and dysfunction, shown to be a consequence of cell free haemoglobin and iron accumulation, and acute kidney injury. These pathological effects of storage in 14 day red cells were partially prevented by red cell rejuvenation; specifically ATP, 2,3 DPG and deformability were restored to values comparable to those of fresh red cells. Rejuvenation had multiple protective effects against inflammation and organ injury in this model. The first was through washing, which reduced red cell microvesicles that accumulated in stored porcine units. This reduced platelet and leucocyte activation, sequestration in lungs and kidneys, and transfusion associated lung injury. The second was through the restoration of the mechanical properties of red cells which reduced the accelerated release of cell free haemoglobin following mechanical washing. This reduced oxidative stress and endothelial dysfunction. The third was through the prevention of tissue iron accumulation in the kidneys of transfusion recipients, and the prevention of transfusion associated acute kidney injury. No adverse effects of transfusion of rejuvenated red cells were reported. The study also explored changes in red cell characteristics between 21-day stored units and rejuvenated units, and found ATP levels to be significantly lower in the stored units compared to the rejuvenated units (data not reported).

**Baboons**

Baboons were the experimental model used by the United States Navy to investigate the role of 2,3-DPG and ATP on red cell function, where rejuvenation was used to model the effects of normal range or supraphysiological 2,3-DPG or ATP values, versus sub-normal values (stored red cells). In early experiments, ABO matched baboon red cells stored in CPD were rejuvenated with PIGPA and re-suspended in a sodium chloride, glucose, phosphate solution until use. In this model, transfusion of rejuvenated red cells was shown to increase the 2,3-DPG, ATP and p50 of circulating blood. This was demonstrated to have a range of benefits in simulated clinical settings including anaemia [40], haemorrhagic shock [41], and hypoxia [42, 43]. Benefits most commonly seen included improved myocardial workload, and cerebral blood flow in anaemic animals [40]. The model was also used to evaluate rejuvenated-freeze-thaw-washed red cells in hypotensive animals. The effects of these cells on tissue oxygenation and cardiac workload were no different to fresh, non-rejuvenated-frozen-thawed-washed red cells despite higher 2,3-DPG and p50 values, although mixed venous oxygen saturations were higher, suggesting more effective tissue oxygen delivery [44].

**Human clinical studies evaluating the effects of rejuvenated red cells (Table 5)**
The first clinical report of administration of rejuvenated red cells to humans was in 1972 [45]. Rejuvenation was performed in 29 day ACD stored red cells with PIGPA solution A (Table 6), followed by glycerolisation, freezing, storage for up to 1 year and then thawing, washing and transfusion within 4 hours. The freeze-thaw recovery was 90% and the 24 hour in vivo post transfusion survival in 5 healthy volunteers was 80%, which meet the requirements of the United States Food and Drug Administration for percentage in vitro RBC recovery [46]. PIGPA Solution A rejuvenated, frozen, thawed, and deglycerolised red cells were also evaluated in a series of clinical trials. Up to 10 red cell units per patient were administered to a cohort of 19 elderly anaemic patients without adverse effects [30]. These cells were also evaluated in two small proof of concept RCTs. Patients undergoing abdominal aortic aneurysm repair and transfused with rejuvenated red cells stored for up to 5 days in CPD had red cells with a higher 2,3-DPG, p50, and a trend towards higher ATP when compared to those transfused with stored or washed and stored red cells [47]. In another RCT, cardiac surgery patients receiving rejuvenated, frozen, thawed, and deglycerolized cells (frozen and stored for 1 year, mean 6 units per patient) had a higher mean 2,3-DPG, p50, cardiac index and oxygen utilisation, as compared to patients receiving stored allogenic or autologous red cells, suggesting that the transfusion of rejuvenated red cells can result in improved myocardial performance [48]. Importantly there were no unexpected adverse events attributed to the transfusion of rejuvenated red cells in any of these clinical trials, or in over 1500 other rejuvenated red cell units transfused, as described in later reports of this technique [12].

**DISCUSSION**

**Main findings**

This systematic review has summarised the evidence relating to the cellular, pre-clinical and clinical effects of red cell rejuvenation and transfusion. In experimental in vivo and in vitro studies the effects of rejuvenation included: improved oxygen offloading due to higher 2,3-DPG; improvements in measures of endothelial function and tissue perfusion, increased deformability and reduced susceptibility to lysis and the release of cell free haemoglobin post mechanical cell washing, all attributable to increased ATP levels. In swine the reduction of red cell derived microvesicles and cell free haemoglobin in the red cell storage supernatant post washing were associated with reduced platelet, leucocyte, and endothelial activation in recipients, as well as improvements in renal, pulmonary and myocardial function when compared to the
transfusion of aged stored red cells. In human clinical studies, rejuvenated red cells were shown to be safe and to improve myocardial function in cardiac surgery patients. Importantly, there were no adverse clinical events recorded with the use of rejuvenated red cells, despite the reported transfusion of over 1500 units of rejuvenated red cells.

Clinical relevance

Uncertainty as to the clinical importance of the red cell storage lesion has led to renewed interest in red cell rejuvenation as a blood safety intervention. Three prospective clinical trials of rejuvenated red cells are underway in patient groups considered at increased risk from transfusion associated morbidity: adult (NCT03167788) and paediatric cardiac surgery (NCT02485366) and those requiring repeated transfusions for sickle cell anaemia (NCT02731157). A further study is assessing changes in VO₂ max before and after transfusion of 2 units of autologous rejuvenated red cells versus stored red cells and after exercise testing (NCT03089047).

Several recent large, prospective, randomised trials have failed to demonstrate a clinical benefit from the use of fresher red cells, stored for 7-13 days versus older red cells >21 days [6, 7, 49]. However, these trials primarily evaluated whether altered storage time has clinical benefits with respect to the transfusion of fresher blood and were not designed to either prove or disprove whether the storage lesion has clinical importance. This is because red cells stored for 7-13 days have already developed a significant storage lesion, with only incremental differences by days 21 to 25 [8]. Indeed, even large registry studies do not show clear differences between patients receiving 7 day versus 21 day old red cells [50]. In contrast, as demonstrated in this review, rejuvenated red cells have many of the attributes of fresh red cells; specifically with respect to 2,3 DPG and ATP levels. The evaluation of rejuvenated red cells in clinical trials is therefore more likely to provide clear evidence as to the clinical importance of depletion of 2,3 DPG and ATP levels as part of the storage lesion. If positive these trials will have important implications for patients, and also for blood services.

Strengths and limitations

This study represents the most current and comprehensive review of red cell rejuvenation to date. It provides a rationale for the continued evaluation of red cell rejuvenation as a clinical intervention. The principal limitation of the review is the heterogeneity of the additive solutions, of the rejuvenation process with respect to thawing, washing, and shelf life at time of rejuvenation, and the definitions of relevant
endpoints reported in the included studies. This heterogeneity prevents quantitative
meta-analyses and limits the conclusions that may be derived from the data. Another
limitation is that some of the animal studies were conducted in healthy animals.
Although rejuvenated red cells demonstrated improved oxygen delivery in vitro and in vivo, as well as reduced post transfusion inflammation, this may not translate into
clinical benefits in critically ill patients or those with multiple comorbidities where
transfusion is common. A further limitation is that the clinical data relating to the use of
red cell rejuvenation may not be relevant to current clinical practice given that these
studies were performed over 30 years ago. The FDA has licensed rejuvesol for sale,
and red cell rejuvenation is described in the Circular of Information for the use of human
blood and blood components [51]. However, there is very little contemporary clinical
data describing its use. In particular, safety reporting in trials undertaken many decades
ago would be considered inadequate by modern standards. Despite these limitations,
on the basis of the available data, we consider there to be enough evidence on the
safety and efficacy of red cell rejuvenation to warrant evaluation in a clinical trial.

Conclusion

A systematic review of studies that have evaluated red cell rejuvenation using a
processing solution rich in pyruvate, inosine, adenine and phosphate restores red cell
properties to similar levels as those of freshly donated red cells. Transfusion of these
red cells reduces organ injury attributable to the red cell storage lesion in experimental
studies in vivo. The review did not identify any significant clinical risks from red cell
rejuvenation, however the clinical benefits of this procedure remain unclear. Ongoing
clinical trials of the process will address this uncertainty and provide new insights into
the clinical importance of the storage lesion.
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