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ORIGINAL PAPER

Rejuvenation solution as an adjunct cold storage solution maintains physiological haemoglobin oxygen affinity during early-storage period of red blood cells

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Abstract

Background Red blood cell (RBC) units accumulate morphologic and metabolic lesions during storage before transfusion. Pyruvate–inosine–phosphate–adenine (PIPA) solutions (Rejuvesol, Biomet, Warsaw, IN) can be incubated with RBC units to mitigate storage lesions. This study proposes a PIPA treatment process, termed cold 'rejuvenation', using Rejuvesol as an adjunct additive solution, to prevent biomechanical storage lesions while avoiding the 1 h PIPA incubation required with standard PIPA treatment. We compared the efficacy of cold to standard 'rejuvenation' in improving metabolic lesions that occur during cold storage of RBCs, without altering function.

Methods Twelve leucoreduced, A-positive RBC units were obtained. Each unit was aliquoted into either control (standard storage), washed (W), standard rejuvenation (SR) or cold rejuvenation (CR) groups, the latter two requiring washing. A volume-adjusted dose of Rejuvesol was instilled into the CR group upon receipt (Day 3). After 15 days of storage, p50, RBC deformability, in-bag haemolysis and mechanical fragility were analysed. 'Any treatment' is defined as W, SR and CR, with comparisons in reference to control.

Results Higher p50s were seen in rejuvenated groups (>30 mmHg vs. <19 mmHg; $P < 0{\cdot}0001$). Any treatment significantly increased elongation index ($P = 0{\cdot}034$) but did not significantly increase in-bag haemolysis ($P = 0{\cdot}062$). Mechanical fragility was not significantly different between groups ($P = 0{\cdot}055$) at baseline, but the control (CTL) group was more fragile after 2 h in a cardiac bypass simulation than any treatment ($P < 0{\cdot}0001$).

Conclusions This study demonstrates that rejuvenation (standard or cold) prevents the leftward p50 shift of storage lesions without detrimental effect on RBC deformity, in-bag haemolysis or mechanical fragility.

Key words: transfusion, erythrocyte additive solution, storage lesion.

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Introduction

Red blood cell (RBC) transfusion is one of the most common medical interventions performed in the United States with broad indications including surgery, trauma and chronic anaemias [1,2]. RBCs are stored on average for 18 days, and up to 42 days, prior to use in allogeneic transfusion [3,4]. During storage, RBCs undergo a deleterious process collectively referred to as the storage lesion. This progressive accumulation of metabolic and morphologic changes alters their structure and function. One of the most notable metabolic changes during storage is depletion of organic phosphates, particularly 2,3-diphosphoglycerate (2,3-DPG); starting within the first days of storage, phosphates begin to be depleted and are virtually undetectable by Day 14 [5,6]. While RECESS [7] and ABLE [8] studies did not demonstrate a difference in clinical outcomes with different storage ages, even the 'fresh' units had been stored for long enough for 2,3-DPG levels, and therefore p50 (partial pressure of oxygen at 50% haemoglobin saturation), to have decreased. This loss of 2,3-DPG results in an increased haemoglobin oxygen affinity and lower p50 [9] demonstrated as a left shift in the oxygen dissociation curve (ODC). This shift in oxygen affinity may place unstable patients, particularly those requiring relatively large RBC volumes, at risk for tissue hypo-perfusion if the left-shift limits the physiological Bohr effect [10–17]. As such, there is a theoretical benefit to maintaining or even intentionally increasing the p50 to improve tissue oxygenation [11]. The established normal range of p50 is 25·9–27·3 mmHg [18].

Several additives or storage alternatives exist to prevent or mitigate the effects of the storage lesion in RBCs [19]. The FDA-approved additive Rejuvesol (Biomet Labs, Braintree, MA) is a pyruvate, inosine, phosphate and adenine (PIPA) solution intended to extend the shelf life of rare RBC units. Rejuvesol has been shown to restore RBC cell levels of adenosine triphosphate (ATP) and 2,3-DPG to or above baseline values, and to normalize cell membrane function and cellular haemoglobin oxygen affinity [19]. Rejuvesol solution is only intended for the extracorporeal rejuvenation of RBC units (Rejuvesol RBC processing solution, Zimmer Biomet, package insert). The use of RBC rejuvenated before 6 days of storage is contraindicated because their high 2,3-DPG levels and low oxygen affinity may excessively increase p50 and impair proper oxygenation of the red blood cells in the lung (Rejuvesol RBC processing solution, Zimmer Biomet, package insert). Rejuvesol solution is recommended after 14 days of liquid storage to restore 2,3-DPG and ATP levels prior to transfusion for immediate use, or to cryopreserve rare blood types or autologous RBCs (Rejuvesol RBC processing solution, Zimmer Biomet, package insert).

The standard PIPA protocol requires RBC unit incubation with the additive at 37°C for one hour, followed by RBC unit washing to remove free Rejuvesol. This time-intensive process is not practical for clinical practice when RBC transfusion must be performed rapidly or when large volumes are required. A fresh disposable was used for each aliquot of split units to avoid cross-contamination. Sampling was done immediately after washing.

In the present study, we propose an alternative to the standard protocol, referred to as 'cold rejuvenation/CR' as described by Gehrke and colleagues in 2018 [19]. The Gehrke study analysed frozen samples generated during this present study, wherein we immediately analyse the function of RBC's. We hypothesized that CR would avoid storage induced increase oxygen affinity and even produce an above-established reference value p50 (right-shift), without adversely affecting deformability and susceptibility to haemolysis compared to untreated RBC units.

Materials and methods

Study design

This in vitro study was exempt from the Duke University Institutional Review Board.

Blood products and preparation of rejuvenated units

Leucoreduced, three-day-old, A-positive RBC units (n = 12) stored in additive solution AS-1 (ADSOL, Fenwal, Lake Zurich, IL) were obtained from the American Red Cross (Durham, NC). Immediately upon receipt, each RBC unit was equally divided by volume into four aliquots using sterile technique and a neonatal/paediatric aliquot bag system (Charter Medical, Winston-Salem, NC). The four aliquots were designated control (CTL), washed (W), standard 'rejuvenation' (SR), and cold 'rejuvenation' (CR) with PIPA solution and stored at 4°C. A volume-adjusted dose of PIPA solution (Rejuvesol, Biomet, Braintree, MA) was instilled into the CR aliquot bag. This volume was calculated based on manufacturer instructions specifying a 1:5 ratio of Rejuvesol to RBC volume. The CR aliquot bag was subsequently returned to storage at 4°C. The blood units were stored, and the Rejuvesol was added in a walk-in refrigerator (~4°C). All units were stored in a walk-in refrigerator (~4°C). No baseline data were collected on Day 3 due to limited volume availability.

Aliquots were retrieved after a total of 15 days of storage. At the time of retrieval, the SR aliquot was instilled, the same volume-adjusted dose of PIPA solution as the CR aliquot, and was subsequently incubated at 37°C for

one hour in a Plasmatherm blood warmer (Barkey GmBH, Leopoldshohe, Germany). Following incubation, the SR aliquot was diluted 1:4 in 0·9% normal saline prior to washing in a Continuous AutoTransfusion System (C.A.T.S; Fresenius Kabi AG, Bad Homburg, Germany) using the High Quality Wash setting [20]. The W and CR aliquots were similarly diluted and washed using new disposable tubing sets for each aliquot to prevent cross-contamination of samples; the CTL aliquot was not subjected to washing. RBC quality assays were run on aliquots from all four groups (CTL, W, SR and CR).

Determination of haemoglobin oxygen affinity (p50)

A Clark oxygen electrode (Hemox Analyzer; TCS Scientific, New Hope, PA) was used to determine the haemoglobin oxygen affinity of each sample by automated tonometry in duplicate. Per manufacturer's instructions, 5 ml of Hemox Buffer, 20 µl of 25% bovine serum albumin and 10 µl of antifoaming agent were mixed with 50 µl of RBCs. Using the Hemox Analyzer, the mixture was exposed to variation in oxygen tension by insufflation of nitrogen gas, while dual-wavelength spectrophotometry at 560 and 570 nm was used to measure changes in oxyhaemoglobin. From this, a p50 and Hill coefficient were derived from the generated oxygen dissociation curve, as previously described [19,22].

Determination of RBC deformability

Deformability was measured by laser diffraction ektacytometry (LoRRca, RR Mechatronics, Hoorn, The Netherlands) via the elongation index of RBCs. The elongation index (EI) is an established parameter to describe the deformability of RBCs [23]. Elongation Index $EI = (L - W)/(L + W)$, where L and W represent the length and width of the ektacytometry diffraction pattern, respectively. Maximum shear stress was 30 kPA. EI is reported as EI max. A volume of 25 µL RBC was added to 5 ml of 6% polyvinylpyrrolidone (PVP) and gently inverted five times to mix. This mixture was manually aliquoted into the rotational cell analyser and run in triplicate using the 'Deformability' program with two cleaning rinse cycles between replicates.

In-bag haemolysis assay

To determine haemolysis occurring during storage, free haemoglobin (FHb) was measured on the supernatant using visible-light spectrophotometry [24] after centrifugation of a sample from each group at 2000 ***g*** for 10 min at 4°C. Per cent haemolysis was calculated from FHb as ((FHb)/(sample Hb)) × 100. HCT was determined by microcapillary sedimentation after centrifugation. The free Hb was only measured after washing in the W, SR and CR groups.

The manual HCT determination was performed as follows: capillary tubes were 75% filled with HPC(A) product, sealed and centrifuged for 10 000–15 000 × g using a microhaematocrit centrifuge (C-MH30, Unico, NJ). The centrifuged tubes were measured for the total height of the sample and the height of the packed red cell layer using a capillary microhaematocrit reader (CMH30, Unico, NJ). The red cell layer height was divided by total sample height to obtain the per cent HCT value. This assay satisfies proficiency testing by method comparison with an automated haematology analyser (Advia 2120i; Siemens Healthcare Diagnostics, Tarrytown, NY) [25].

Mechanical fragility assay

Transfused blood in patients on extracorporeal circuits such as ECMO or cardiopulmonary bypass/CPB undergoes significant mechanical stress [22]. The mechanical fragility of RBCs was evaluated with a model CPB circuit, using an Alaris infusion pump (CareFusion, San Diego, CA) with compatible tubing (BD) drawing from the reservoir of a 20 ml syringe and emptying back into the reservoir through a 25-gauge needle (Fig. 1). This small needle diameter and flow rate were chosen to maximize the mechanical shear stress within the circuit. Samples were diluted in normal saline to 25% haematocrit, similar to that seen during CPB, before duplicate 2-h runs in the model CPB circuit with sampling at time zero, one and 2 h. Supernatant FHb was measured using visible-light spectrophotometry [24].

Statistical analysis

Mixed models were used to account for repeated measurements of each RBC unit. For each of the three outcomes (p50, elongation index, in-bag haemolysis), a mixed linear regression model was fit with a fixed effect for treatment group and a random intercept for RBC unit. The significance of the overall effect of treatment group was assessed using a type III test of significance. Treatment group is defined as W, SR or CR, and all statistical analyses are comparing these treatment groups to the control.

For the Mechanical Fragility tests, a mixed linear regression model was fit with fixed effects for treatment group and time as well as a random intercept for RBC unit to account for repeated measurements. An

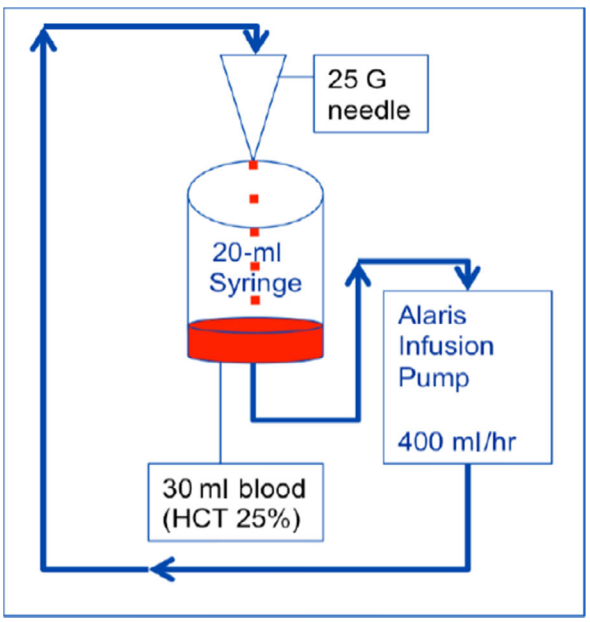

Fig. 1 The four groups of red blood cells were subject to mechanical stresses using the following circuit. This approximates the shear stress encountered during cardiopulmonary bypass or extracorporeal mechanical circulatory support. [Colour figure can be viewed at wileyonlinelibrary.com]

interaction between treatment group and time was added to the model. The significance of overall effects of treatment group, time and treatment group-by-time interaction was assessed using type III tests of significance. In all tests, a *P*-value less than 0·05 was considered statistically significant. Average values are presented as mean ± standard deviation. All statistical analyses were performed using R, version 3.5.0 (https://cran.r-project.org/).

Results

For the three groups (CR, SR and W), we compared the effects of RBC treatment to CTL methodology, to determine if the CR method was significantly different compared to CTL.

RBC aliquots subjected to rejuvenation solutions demonstrate higher p50 values

In line with the mechanism of action of the PIPA solution, higher average p50 values were observed in the rejuvenated CR and SR groups compared to non-rejuvenated W and CTL groups (Table 1). The CR and SR groups significantly increased the p50 compared to CTL. As detailed in Table 1, with regard to p50, CR had a significantly higher p50 compared to CTL, but, despite them both being higher than control, the p50 after CR was lower than after SR (31·4 vs. 35·2; $P < 0·001$).

Table 1 Oxygen affinity, deformability and in-bag haemolysis for treatments groups (CR, SR and W) compared to control (CTL)

Oxygen affinity (P50)		
Treatment group	**P50 (mmHg)* ± SD**	***P*-value**
CR	31·4 ± 2·6	<0·0001
SR	35·2 ± 1·5	<0·0001
CTL	18·1 ± 1·3	N/A
W	16·7 ± 1·1	0·0023
RBC deformability* (Elongation Index/EI)		
Treatment group	**EI ± SD**	***P*-value**
CR	0·627 ± 0·02	0·2431
SR	0·637 ± 0·02	0·0132
CTL	0·619 ± 0·04	N/A
W	0·637 ± 0·02	0·0112
In-bag haemolysis		
Treatment group	**Per cent (%)* ± SD**	***P*-value**
CR	0·19 ± 0·05	0·1235
SR	0·19 ± 0·05	0·0142
CTL	0·15 ± 0·06	N/A
W	0·18 ± 0·03	0·0270

CTL, control; CR, cold rejuvenation; SR, standard rejuvenation; W, washed.
*Mean ± SD

Rejuvenation does not significantly impact in-bag RBC haemolysis

There was no significant difference in the CR, SR and W treatment groups for in-bag % haemolysis as compared to CTL ($P = 0·06$) and no significant difference between the CR and SR groups for in-bag haemolysis ($P = 0·4028$). These non-significant increases in the % haemolysis after each washing manipulation of these units of 15-day storage age are well below the AABB mandated acceptable limit of 0·8%. Rates of 0·2–0·4% are consistent with other studies [26].

Rejuvenated and washed RBC units increase RBC deformability

As detailed in Table 1, the overall treatment group significantly increased the elongation index when compared to CTL ($P = 0·03$). Between groups, only the SR and W groups significantly increased EI when compared to CTL ($P = 0·013$ and $P = 0·011$, respectively).

Cold rejuvenation positively impacts RBC mechanical fragility

The CPB circuit model induced shear stress-related haemolysis of RBCs as indicated by significant elevations in FHb over time from t(0) to t(2) in all RBC unit groups ($P < 0{\cdot}0001$ for all) (Figure 2). Following the trends in Figure 2, after one and two hours of exposure to the stress of the circuit, the washed groups (W, CR and SR) all showed less haemolysis than control, but the only significant difference in mechanical fragility (stress induced haemolysis) across all groups was seen with CR when compared to CTL ($P = 0{\cdot}0339$).

Discussion

The results of this present study demonstrated that cold or standard rejuvenation of RBCs stored for 15 days is able to restore the p50 to above reference levels, in contrast to the abnormally low levels observed after relatively short-term storage. A primary concern was whether the process of rejuvenation and washing in a cell saver (consistent with the practical concept of 'bedside washing') imparted a sub-lethal injury to the RBCs that would render them more susceptible to haemolysis thereby negating the benefits of rejuvenation. However, evidence from exposure to prolonged shear stress from circulating in the model CPB circuit in this present study is not consistent with haemolysis and sub-lethal RBC injury.

Rejuvenation solutions support p50 values above established reference ranges

Addition of PIPA solution during refrigerated storage (CR) was effective at preventing the decrease in p50 seen with standard refrigerated storage, producing a p50 above the normal reference range, albeit significantly lower (31·4 vs. 35·2 mmHg, $P < 0{\cdot}0001$) than the standard procedure of incubation with PIPA solution (SR). In contrast, control and washed samples had a significantly diminished p50 of 18·1 and 16·7 mmHg, respectively, as expected with refrigerated storage. [5,6] This finding of above reference range p50 values in PIPA treated RBCs may be used clinically to prevent an acute drop in p50, inferred by decreased 2,3DPG levels, that can be seen after larger volume transfusions [27], which theoretically will reduce tissue oxygen delivery [27]. While the washing step is responsible for removing metabolic storage by-products from RBCs, such as procoagulant microparticles, the cold and incubated PIPA treatment processes appear to benefit ATP restoration and 2,3-DPG levels while reactivating glycolysis [19].

RBC deformability is altered by overall treatment group effect

When using the EI max parameter, a measure of deformability, there was a difference between groups. Elongation index has been shown to become

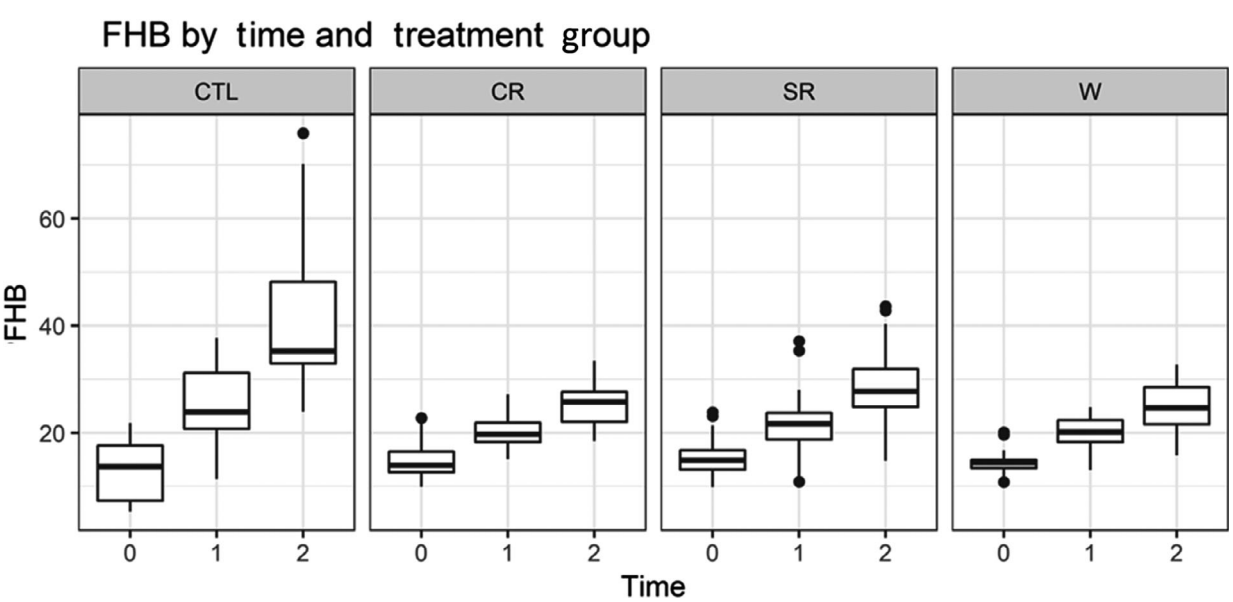

Fig. 2 Cell-free haemoglobin (FHb) release as a measure of mechanical fragility. The time exposed to the model circuit increased FHb in all groups ($P < 0{\cdot}0001$), while there was more FHb released in the control group ($P = 0{\cdot}055$). Abbreviations: CR, cold rejuvenation; SR, standard rejuvenation; CTL, control; W, washed.

abnormally decreased during storage, and the level of reduction in deformability is associated with the period of the storage [21].

Storage of blood causes irreversible changes in biochemical and biomechanical properties of red blood cells. RBCs become metabolically depleted as the energy levels decrease [21]. The geometric characteristics of the cells are impaired, and they become more rigid. Their ability to change shape in response to mechanical stresses is further diminished, though this unique ability of RBCs to deform is essential for maintaining a healthy microvasculature [21].

In this present study, the overall treatment group had significantly higher EImax (30 kPa) than the CTL, indicating greater deformability of the RBC when exposed to high shear stress, as previously described [28].

There is no difference in mechanical fragility between treatment groups

In-bag per cent haemolysis, all of which was considerably below the acceptable maximum of 0·8% [22], did not demonstrate significant differences between groups. Mechanical fragility, as determined by inducing mechanical shear stress in the model CPB circuit, was demonstrated by an increase in FHb over time exposed to the model circuit. The significant increase in haemolysis with time indicates that our model CPB circuit effectively induced mechanical stress. While the effectiveness of this model is apparent, it may also represent a limitation of this study as it may induce a level of shear stress that RBCs, regardless of PIPA treatment, cannot withstand. With a wider gauge needle inducing less shear stress, differences between RBC groups may become apparent. Despite an insignificant increase in plasma free haemoglobin after the wash procedure (with or without rejuvenation), the response to the stress of CPB was significantly blunted in these three groups (W, SR and CR).

Gehrke and colleagues have shown semi-quantitative restoration of 2,3-DPG levels after PIPA treatment [19]; this is in keeping with the present p50 findings. This indicates that after 15 days of storage, with Rejuvesol added at Day 3 as an adjunct additive solution, AS-1 or 3 additive solution may become similar to PAG3M or AS-7 with regard to maintenance of physiological 2,3-DPG levels. By avoiding the incubation step, this approach may offer a practical alternative to the standard PIPA treatment process to above reference range p50 in the more commonly used AS-1 and AS-3 additives, if so desired for certain units or populations. Gehrke and colleagues reported regeneration of ATP using CR and SR methodology. The Gehrke study effectively reported the metabolomics and energy regeneration of these samples, while our present study reports the benefit of using rejuvenation solutions without altering physical RBC properties.

Novel additive solutions are not widely available or FDA-approved in the United States, and should we want to normalize p50, we would need to normalize prior to transfusion. The field of transfusion medicine warrants further studies assessing clinical applications, although the purpose of this experiment was to evaluate a simpler rapidly deployable unit.

The addition of cold rejuvenation avoids the one-hour incubation process.

Limitations

This study observed the effect of storage, and 15 days was chosen as the end-point. Further work would include looking at the effect of cold rejuvenation after different storage durations. The limitations of this study include the sample size and only having a single time-point measured. This was because our experimental design required at least 60 ml of PRBCs to wash in our 'bedside' device in order to keep our study directly clinically relevant. By dividing each RBC bag into four equal-volume aliquots, we did not have enough volume to assess more than one time-point. Furthermore, we sought to treat each unit with the 4 described methods due to the marked interindividual variability in susceptibility of donor blood to haemolysis.

Conclusion

Cold rejuvenation offers a practical and rapidly testable approach to support p50 above normal reference ranges in RBCs for clinical use, which theoretically may offer a physiological advantage. Of particular clinical interest is the possibility of increasing end-organ oxygen availability in patients with anaemia or compromised cardiac function via an increase in systemic p50. Human trials are needed to better understand the potential clinical benefits to modulating p50.

Conflict of interest

This study was funded by Zimmer Biomet, manufacturers of Rejuvesol. IJW is supported by the NHLBI: R01HL121232-05.

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