Original article

Pathogen reduced red blood cells as an alternative to irradiated and washed components with potential for up to 42 days storage

Linda Larsson[1,2], Sara Ohlsson[2], Theresa Neimert Andersson[1,2], Emma Watz[1,3], Stella Larsson[2], Per Sandgren[1,2], Michael Uhlin[1,2]

[1]*Department of Clinical Science, Intervention and Technology (CLINTEC), Karolinska Institutet, Stockholm, Sweden;*
[2]*Department of Clinical Immunology and Transfusion Medicine, Karolinska University Hospital, Stockholm, Sweden;*
[3]*Department of Clinical Immunology and Transfusion Medicine, Uppsala University Hospital, Uppsala, Sweden*

Background - The urgency of maintaining a safe and adequate blood supply is increasing. One approach to ensure a sufficient supply is to limit the outdating frequency of blood components. Pathogen inactivation technology was developed primarily to increase safety by preventing transmission of infectious diseases. The Intercept Blood System for pathogen reduction of red blood cells (RBC) has additional benefits such as inactivation of leucocytes and removal of plasma and storage debris through centrifugation. Irradiation and automated washing are detrimental to the RBC membrane and often implicate shortened shelf-life. We aimed to assess whether pathogen inactivation can replace RBC irradiation and washing to avoid shelf-life reduction.

Materials and methods - RBC concentrates (No.=48) were pooled-and-split into four study arms, which underwent pathogen inactivation treatment, irradiation, automated washing or no treatment (reference). RBC quality was evaluated during 42 days by assessment of storage lesion. Washing efficacy was defined by IgA and albumin reduction.

Results - Pathogen reduced RBCs had similar membrane preservation to reference RBCs (hemolysis, microvesicles and extracellular potassium ions), whereas the RBCs were negatively impacted by irradiation or automated washing. ATP increased substantially post-pathogen inactivation, while 2,3-DPG decreased. Pathogen inactivation considerably reduced albumin and IgA, though slightly less efficiently than automated washing.

Discussion - RBCs exhibit superior membrane preservation after pathogen inactivation treatment, compared to both irradiation and automated washing. This suggests that replacement is possible, even though the plasma reduction protocol could be further optimised.

Replacement of irradiated and washed RBC concentrates with pathogen reduced RBC concentrates storable up to 42 days would be advantageous for both the blood supply and patient safety.

Keywords: *red blood cells, pathogen inactivation, pathogen reduction, blood components, blood supply.*

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Correspondence: Linda Larsson
e-mail: linda.larsson@ki.se

INTRODUCTION

Pathogen inactivation (PI) is a well-established measure to increase blood safety. The principle of PI technology is a pro-active treatment that inhibits replication of any present

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pathogens via interaction with their DNA/RNA. Through this approach, the prevalence of transfusion-transmitted infectious diseases (TTID) can be greatly reduced. Residual leucocytes, instigators of transfusion-associated graft-versus-host disease, are concomitantly inactivated by the same principle[1-5]. Blood components treated with PI measures are generally referred to as "pathogen reduced" (PR).

Cerus Corporation (Concord, CA, USA) is developing a PI system for red blood cells (RBC), Intercept, that is based on irreversible nucleic acid cross-linking with amustaline (S-303); a pH driven reaction. Amustaline and glutathione (GSH; quencher of non-specific nucleophile reactions) are added to the RBC concentrate (RCC), and after incubation, the non-reactive degradation product S-300 is removed through centrifugation. Afterwards, the RBCs are resuspended in new additive solution (AS)[1,6]. The inactivation capacity and clinical utility of Intercept has been demonstrated in several studies[2-4,7].

The main commercial focus of PI is to ensure increased safety in terms of transmission of blood-borne pathogens. Although relevant enough, and despite demonstrations of bacterial growth in refrigerated RBCs[8], the need for PI treatment of RBCs is sometimes difficult to justify in countries where the climate is cold, prevalence of TTID is low and hygiene and processing conditions are good. However, the potential benefits of PI for the RBC storage quality after secondary processing might help justification.

Irradiation is the long-standing technique of choice for inhibition of residual lymphocyte proliferation[9,10]. Automated washing with ACP 215 (Haemonetics Corp., Braintree, MA, USA) was a milestone in the reduction of plasma proteins, IgA and storage lesion debris in RCCs[11,12]. Both these techniques, although efficient in their intended purpose, come at the cost of increased RBC membrane deterioration, implicating up to 28 days reduction of RCC shelf-life. Shorter shelf-life may increase wastage. Therefore, RCCs are often irradiated or washed on demand, which increases the risk of transfusion delays, especially at remote hospital blood banks.

The urgency of maintaining acceptable RBC quality and long shelf-life is growing, as Europe is facing an oncoming increased demand and decreased availability of blood due to a decreasing donor population and longer life expectancy[13,14]. Concomitantly, there are increasing demands on strengthened preparedness to ensure the blood supply in emergency situations[15], which might be even more important if the transition to non-di(2-ethylhexyl) phthalate (DEHP) blood bags necessitates reduced RCC shelf-life to ensure sufficient quality, especially after secondary processing[16-18].

PR-RBCs demonstrate *in vitro* quality and *in vivo* survival equal to conventional RBCs[7,19,20]. We aimed to confirm this, and also assess whether PR-RBCs have superior quality to irradiated and automated-washed RBCs during 42 days storage. Furthermore, we wanted to compare the washing efficacy of the PI procedure to automated washing.

If results prove satisfactory, irradiated and washed RCCs could potentially be substituted by one joint component that could be prepared in advance and stored as long as a conventional RCC. This would facilitate optimization of the blood supply while increasing blood safety.

MATERIALS AND METHODS

Collection and processing

A total of 48 RCCs were derived from 450 mL whole blood (WB) in citrate-phosphate-dextrose following Karolinska standard operating procedures. Key processing steps included collection day separation (RBCs, plasma and buffy coat) using Macospin (3,130 × *g*, 11 minutes) and MacoPress Smart Revo (both Macopharma, Mouvaux, France), addition of 100 mL saline-adenine-glucose-mannitol (SAGM) AS, and RBC leucofiltration.

The RCCs were pooled randomly four-and-four, then split into four equal study arms using the original RCC bags: PR, irradiation, automated washing and reference; No=12 each. Before split, each pool was analysed for baseline values. The RCCs were placed in 2-6°C storage within 8 hours of collection.

PI was executed on day (d) 1-2 following the manufacturer's specifications (Cerus Corp.)[6]. In short, the manufacturer's 0.2 µm hydrophobic air filter set and processing set (containing 140 mL processing solution: adenine, mannitol, sodium citrate, mono- and disodium phosphate) were sterile connected to the RCC. GSH (16.0 mL; 600 mM), the RCC and amustaline (18.0 mL; 6 mM) were added to the processing set during mixing. The mixture was held at 20-25°C for 18-24 hours. Afterwards, the bag was centrifuged (Macospin; 4,194 × *g*, 8 minutes,

22°C) to remove the supernatant/amustaline metabolites. After extraction, new SAGM (90 mL) was added. The PR-RCC was sampled, then returned to cold storage within 26 hours from processing start.

X-ray irradiation (Raycell Mk2, Best Theratronics, Ottawa, Canada; 25-50 Gy) was executed on d14 post-collection, reflecting worst-case scenario (maximum pre-irradiation RCC age; Swedish national guidelines[21]). Automated washing was performed d14 post-collection for similar reason, using ACP 215 (Haemonetics Corp.; bowl volume 275 mL) and SAGM as post-processing AS. The reference RCCs were not processed further.

Sampling

In addition to d0 baseline, all RCCs were sampled on d2 (post-PI), d7, d14, d21, d28, d35 and d42. Irradiated and automated-washed RCCs were sampled twice on d14: before and after the interventions; PR and reference RCCs were sampled simultaneously as the pre-processing sampling. 40 mL sampling bags (VSE0000Y, Macopharma) were used for all sampling.

Analysis

The impact of the different processing interventions on RBC storage was analysed using a combination of methods assessing changes in RBC membrane integrity, metabolism and morphology, a.k.a storage lesion.

Hematocrit (Hct; %), hemoglobin concentration (Hb_{RCC}; g/unit and g/L) and mean corpuscular volume (MCV; fL) were analysed using Swelab Alfa Plus Basic hematology analyzer (Boule Diagnostics AB, Spånga, Sweden). Hemolysis was assessed by measuring free supernatant hemoglobin ($Hb_{supernatant}$; g/L) with HemoCue plasma/low hemoglobin photometer (Radiometer Medical ApS, Brønshøj, Denmark) and calculating hemolysis (%) as (100-Hct) × $Hb_{supernatant}$ (g/L) / Hb_{RCC} (g/L).

Extracellular potassium ions (K^+; mmol/L), reflecting both membrane integrity and metabolic function, and the additional metabolic markers pH, glucose (mmol/L) and lactate (mmol/L) were measured on ABL 800 Flex blood gas analyser (Radiometer Medical ApS). To compensate for differences in Hct, the concentrations were re-calculated: (mmol/L × [1-Hct]) / Hb_{RCC} (g/L) × 1,000 = mmol/mg Hb.

Adenosine triphosphate (ATP; µmol/g Hb) was extracted by trichloroacetic acid and measured by luminometry (Orion Microplate Luminometer, Berthold, Pforzheim, Germany) using ATP Kit SL (BioThema, Handen, Sweden).

2,3-diphosphoglycerate (2,3-DPG; µmol/g Hb), impacting oxygen dissociation, was extracted by perchloric acid and neutralising potassium carbonate; then analysed spectrophotometrically (SpectraMax, Molecular Devices, San Jose, CA, USA) with 2,3-DPG test kit 10148334001 (Roche Diagnostics, Mannheim, Germany). Due to kit shortage, the sample size was reduced to No.=6, rationed as d0: pool, d2: PR, d7: PR and reference (reference was considered representative of irradiated and automated-washed RCCs as these remained untreated at d7) and d14: all study arms (post-intervention only).

RBC morphology was analysed indirectly by assessing the count and surface characteristics of shed RBC microvesicles (RMV) via flow cytometry (CytoFLEX, Beckman Coulter, Brea, CA). In short, RMVs were suspended in the RCC supernatant by single centrifugation (2,000 × *g*, 10 minutes, 4°C) and stored in ≤ −70°C. 50 µL thawed samples were pipetted into TruCount tubes and incubated with 5 µL PE-Cy7 anti-CD235a, 5 µL BV421 anti-CD47 (d2, d21-d42, unit 9-12 only), 3 µL Alexa Fluor 647 anti-Annexin V and 100 µL buffer (Annexin V: Bio-Legend, San Diego, CA, USA; all others: BD Biosciences, San Jose, CA, USA) for 20 minutes. The microvesicle population was gated as ≤0.9 µm Ø) using sizing beads (Megamix; BioCytex, Marseille, France). Gates were set for RMVs as CD235a positive events (glycophorin A, marking RBC origin) within the microvesicle gate; and for Annexin V (externalised phosphatidylserine) and CD47 positive events within the RMV gate. Number of RMVs per µL supernatant was calculated as (number of events CD235a / number of events TruCount beads) × (number of beads in tube / sample volume). Annexin V and CD47 positive RMVs were calculated as % of total number of RMVs. Gating strategy is presented in *Online Content*, **Figure S1**.

To compare the washing efficacy between automated washing and PI centrifugation, IgA (mg/L; units 1-8 only) and albumin (g/unit) were measured on d0 (pool), d2 post-PI and d14 post-automated washing. IgA_{pool} was measured using turbidimetry (Optilite, Binding Site, Birmingham, UK); $IgA_{post\text{-}intervention}$ using an inhouse enzyme-linked immunosorbent assay (Clinical Immunology, Karolinska University Laboratory, Stockholm, Sweden). Albumin analysis was performed by Clinical Chemistry, Karolinska University Laboratory.

To exclude influence by leucocytes or bacteria, white blood

cells were counted with ADAM-rWBC (NanoEnTek, Seoul, South Korea) post-WB processing, and bacteriological screening was performed at storage end (Clinical Microbiology, Karolinska University Laboratory).

Statistical analysis

D'Agostino-Pearson normality test was performed to justify the use of mean ± standard deviation for data presentation. Significance was tested using repeated measures two-way ANOVA with Holm-Šídák's multiple comparisons test between study arms. Paired t-test was used to test differences between sampling occasions within a single study arm. For 2,3-DPG specifically, d2 and d7 were tested with paired t-test and d14 with two-way ANOVA. GraphPad Prism v.9.3.1 for Windows (GraphPad Software Inc., La Jolla, CA, USA) was used for all statistical computation.

Ethical considerations

All WB units were collected from voluntary, non-remunerated, consenting donors. As all material used in the study was fully anonymised, no further ethical approval was warranted.

RESULTS

No major impact on hematology parameters

PR-RCCs had marginally higher Hct than reference RCCs ($p<0.01$) at d42, but Hb remained similar to reference. Irradiation increased Hct from d28 onwards ($p<0.01$), whereas automated washing substantially decreased Hct and Hb ($p<0.001$) due to the nature of the procedure[22,23] (**Table I**, **Figures 1A-C**). MCV increased similarly in PR-, reference and automated-washed RCCs, even though automated washing momentarily detained the process (d14-21). Irradiation further accelerated the MCV increase (**Table I**, **Figure 1D**).

Comparable membrane preservation to conventional RCCs

No difference could be demonstrated between PR-RCC and reference RCC hemolysis at storage end, even though

Table I - *RBC quality at storage beginning and end*

Analysis parameter	Day	Pathogen reduction	Irradiation	Automated washing	Reference
Hemoglobin (g/L)	2 42	183±4 184±3^{b}	183±3 184±3^{d}	182±4 148±3^{bdf}	182±4 183±3^{f}
Hemoglobin (g/unit)	2 42	45±2 33±1	47±1 32±1^{e}	47±2 32±2^{f}	46±2 34±1^{ef}
Hematocrit (%)	2 42	59.6±0.8 62.2±0.9^{abc}	58.9±0.5 66.4±1.4^{ade}	58.7±0.8 50.4±1.3^{bdf}	58.8±0.8 63.4±1.2^{cef}
Hemolysis (%)	2 42	0.04±0.02^{abc} 0.21±0.02^{ab}	0.01±0.01^{a} 0.58±0.07^{ade}	0.01±0.01^{b} 0.37±0.04^{bdf}	0.01±0.01^{c} 0.20±0.02^{ef}
RMV, count (× 10^{3}/µL supernatant)	2 42	0.79±0.46 7.50±1.42^{abc}	0.86±0.29 39.39±6.02^{ade}	0.97±0.39 12.31±2.35^{bdf}	0.90±0.36 9.99±2.97^{cef}
RMV microvesicles, phosphatidylserine (%)	2 42	95.5±1.4 66.3±5.1^{ab}	94.2±2.4 39.8±9.7^{ade}	95.7±2.4 74.2±7.9^{bd}	94.8±2.1 67.7±9.7^{e}
RMV microvesicles, CD47 (%)	2 42	96.8±1.3 68.5±6.8^{ab}	92.5±2.9 36.3±11.2^{ade}	97.8±1.0 99.3±1.0^{bdf}	95.3±2.5 69.8±9.0^{ef}
Extracellular K^{+} (mmol/mg Hb)	2 42	2.5±0.3 85.5±3.0^{abc}	15.0±0.9 112.9±4.5^{ade}	15.2±1.0 73.1±2.7^{bdf}	15.2±0.8 92.1± 3.3^{cef}
ATP (µmol/g Hb)	2 42	8.7±0.5^{abc} 5.1±0.9^{abc}	6.0±0.5^{a} 3.4±0.6^{a}	6.0±0.4^{b} 3.6±0.6^{b}	6.0±0.8^{c} 4.0±0.8^{c}
pH 37°C	2 42	6.753±0.016^{abc} 6.264±0.012^{abc}	6.929±0.026^{a} 6.384±0.014^{ad}	6.929 0.024^{b} 6.224±0.012^{bdf}	6.932±0.025^{c} 6.384±0.012^{cf}
Glucose (mmol/mg Hb)	2 42	57.1±2.1^{abc} 34.6±2.2^{ab}	63.8±2.0^{a} 30.4±2.3^{ade}	63.3±2.8^{b} 70.9±4.8^{bdf}	64.6±2.5^{c} 33.4±2.4^{ef}
Lactate (mmol/mg Hb)	2 42	9.8±0.5^{abc} 41.8±1.6^{abc}	11.6±0.7^{a} 49.3±2.5^{ade}	11.6±0.8^{b} 39.4±2.1^{bdf}	11.5±0.7^{c} 54.4±2.1^{cef}
Mean corpuscular volume (fl)	2 42	93.6±2.1 97.2±2.4^{a}	93.0±2.0 104.0±2.5^{ade}	92.9±2.1 98.7±2.4^{d}	93.0±2.0 99.2±2.4^{e}

Data is presented as mean ± SD. No.=12 per study arm except 2,3-DPG (No.=6); CD47 (No.=4). Significant differences ($p<0.05$) are shown as: ^{a}Pathogen reduced *vs* Irradiated; ^{b}Pathogen reduced *vs* Washed; ^{c}Pathogen reduced *vs* Reference; ^{d}Irradiated *vs* Washed; ^{e}Irradiated *vs* Reference; ^{f}Washed *vs* Reference.

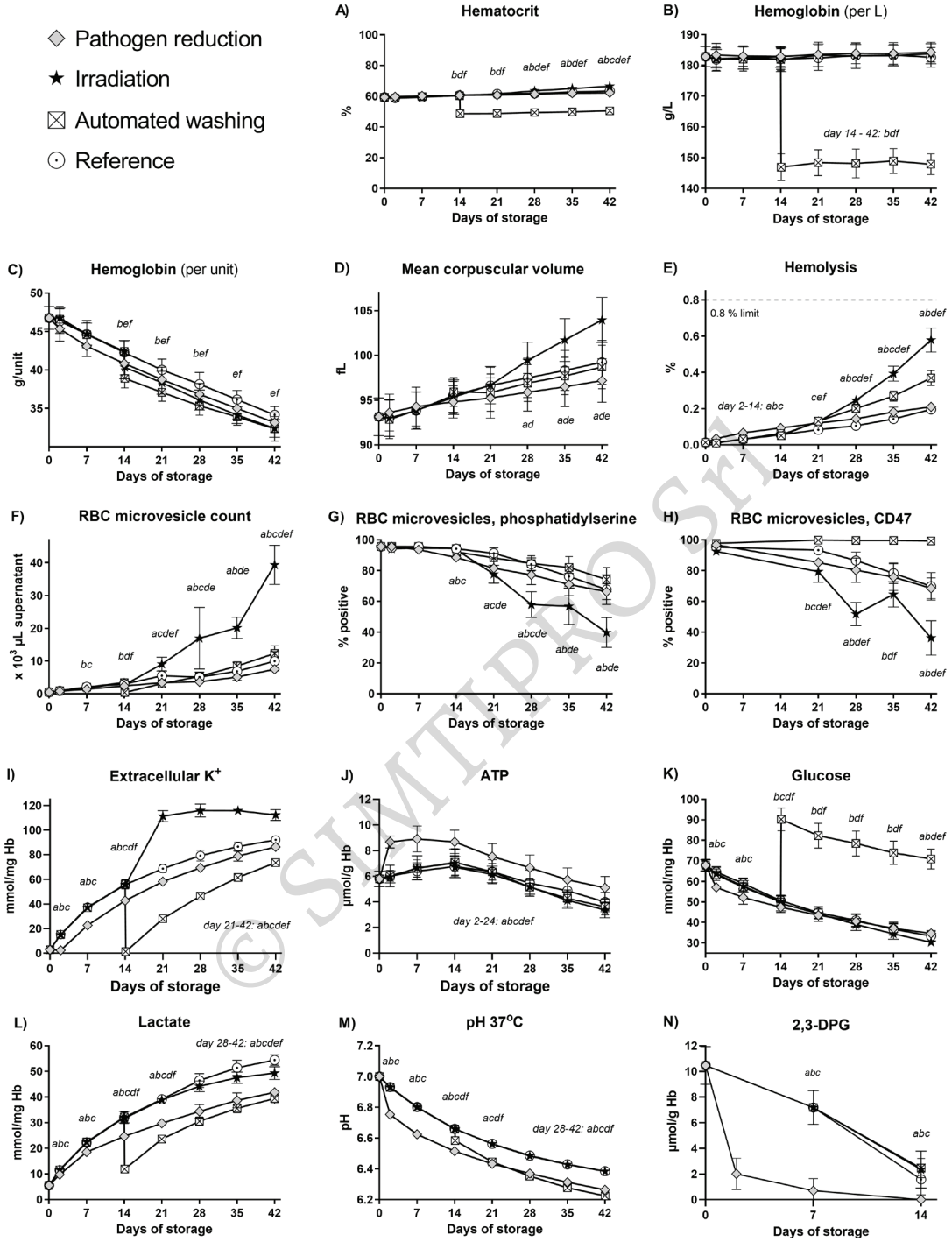

Figure 1 - Storage-related changes in levels of A) hematocrit, B) hemoglobin/l, C) hemoglobin/unit, D) mean corpuscular volume, E) hemolysis, F) red blood cell (RBC) microvesicle count, G) RBC microvesicle phosphatidylserine surface expression, H) RBC microvesicle CD47 surface expression, I) extracellular potassium ions (K^+), J) adenosine triphosphate (ATP), K) glucose, L) lactate, M) pH and N) 2,3-diphosphoglycerate (2,3-DPG)

Grey diamond (◇) = pathogen reduced, black star (★) = irradiated, crossed square (☒) = automated washed and circle with dot (⊙) = Reference. Dotted line in (e): EDQM limit 0.8%. Values are displayed as mean ± standard deviation. No.=12 except 2,3-DPG (No.=6) and CD47 (No.=4).
Significant differences ($p<0.05$): *a* - pathogen reduced *vs* irradiated, *b* - pathogen reduced *vs* automated washed, *c* - pathogen reduced *vs* reference, *d* - irradiated *vs* automated washed, *e* - irradiated *vs* reference, *f* -automated washed *vs* reference.

PR-RCCs had marginally higher levels during early storage (p<0.01, maximum difference 0.04%). PR-RCC storage end hemolysis 0.21±0.02% equalled about ¼ of the European maximum limit 0.8%[24]. While PR-RCCs remained generally unaffected by treatment, the hemolysis rate was substantially accelerated after both irradiation and automated washing (**Table I**, **Figure 1E**).

The lowest d42 RMV concentration was found in PR-RCCs ($p<0.05$); even though washing was executed at a later time (d14) for automated-washed RCCs. The post-irradiation RMV concentration was 5-fold higher than post-PR on d42 (**Table I**, **Figure 1F**).

RMV surface phosphatidylserine decreased similarly over time for PR, automated-washed and reference RCCs, whereas irradiated RCCs decreased substantially faster (<40% on d42, as compared to 66-77% in the other study arms; p<0.001). CD47 decreased in conformity with phosphatidylserine in all study arms except automated washing, where levels remained >97% until storage end (**Table I**, **Figures 1G-H**).

Extracellular K^+ was substantially reduced both by PR-processing and automated washing. The concentration in PR-RCCs increased with similar rate as reference, while the increase rate per mg Hb was faster after automated washing and immediately after irradiation (**Table I**, **Figure 1**).

Improved ATP levels and altered metabolism

ATP increased directly after PR and remained >1 µmol/g Hb higher than the other study arms throughout storage. The glucose concentration was reduced post-PR (p<0.001) but consumption rate was slower. From d28, a PR intervention-related difference was no longer discernible. Correspondingly, lactate accumulated slower after PR, both compared to reference and automated washing. pH fell approximately 0.150 post-PR, which remained throughout storage (p<0.001; **Table I**, **Figures 1J-M**).

Premature 2,3-DPG depletion

2,3-DPG dropped after PR treatment; from 10.5±1.5 (d0) to 2.0±1.2 µmol/g Hb (p<0.001; d2). On d7, 33% of the PR-RCCs were depleted, while reference remained at 7.2±1.3 µmol/g Hb (p<0.001). On d14, all PR-RCCs were depleted while the means of the other arms remained at 1.6-2.5 µmol/g Hb (ns; **Figure 1N**).

Slightly less efficient plasma reduction

Albumin content was reduced 96.0% post-PR and 98.4% post-automated washing compared to d0 baseline. IgA was reduced similarly; 94.4% (PR) and 99.8% (automated washing) (**Table II**, **Figure 2**).

Satisfactory RCC processing

All RCCs contained <1×10^6 residual leukocytes per unit. Bacterial growth was <5 colony forming units/mL at storage end in all RCCs.

DISCUSSION

This study assessed RBC quality after PI treatment, focusing specifically on comparison to irradiation and automated washing with the aim to consider a future substitution of both these treatments. Our results suggest that such a replacement might indeed be achievable.

Table II - *Efficacy of plasma removal: comparison between pre-treatment baseline, PR and automated washing*

IgA			
Unit	**Pool**	**Pathogen reduction**	**Automated washing**
mg/L RCC	50.00±10.54	2.79±0.90***	0.08±0.03***
mg/unit	13.48±2.84	0.70±0.22***	0.02±0.01***
washing efficacy (%)	N/A	94.4	99.8
Albumin			
Unit	**Pool**	**Pathogen reduction**	**Automated washing**
g/L RCC	0.956±0.112	0.038±0.007***	0.015±0.007***
g/unit	0.244±0.030	0.010±0.002***	0.004±0.002***
washing efficacy (%)	N/A	96.0	98.4

Data is presented as mean ± SD. IgA, No.=8; albumin, No.=12. Significant differences between pathogen reduction and automated washing are shown as *** p<0.001.

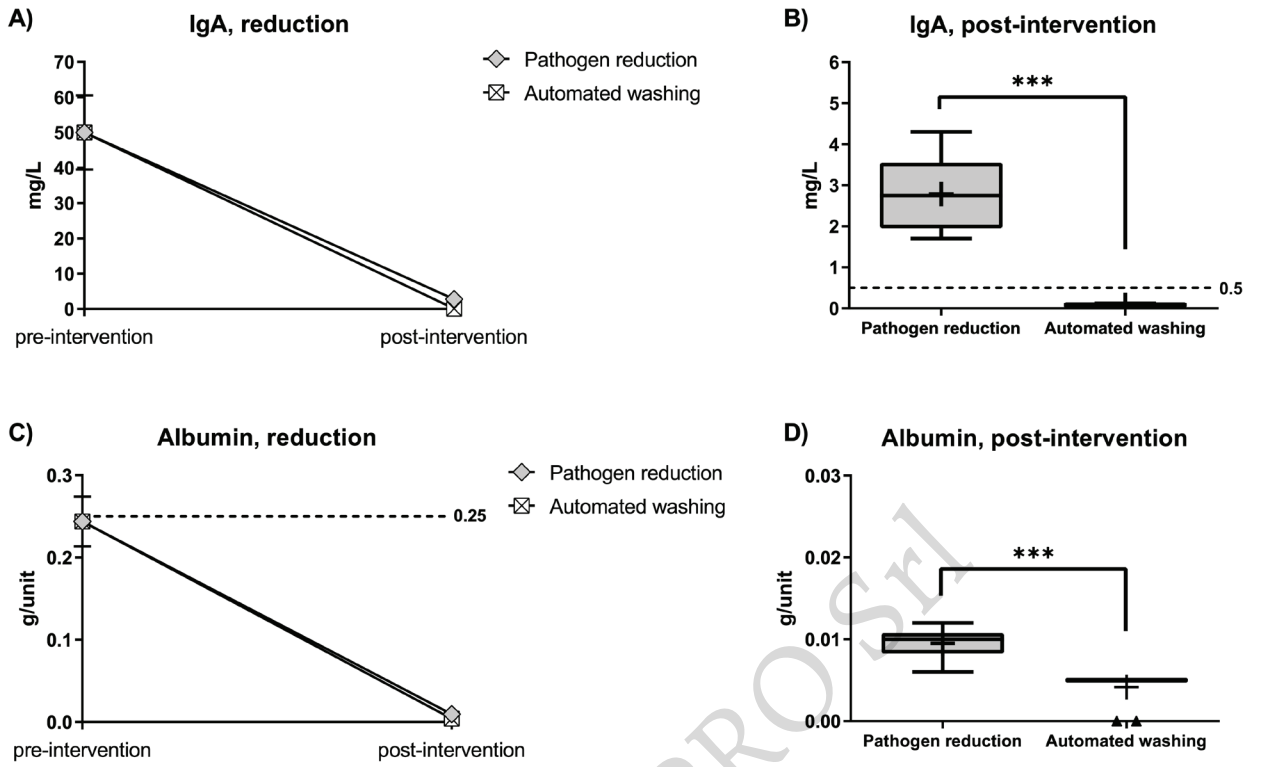

Figure 2 - A-B) Reduction of IgA in pathogen reduced and automated-washed red blood cell concentrates (RCC). Dotted line in B): AABB post-wash limit for IgA: 0.5 mg/L. C-D) Reduction of albumin in pathogen reduced and automated-washed RCCs. Dotted line in C): approximated post-wash limit for albumin (0.25 g/unit), estimated from 50% of EDQM limit 0.5 g/unit for total protein content. Values in A) and C) are displayed as mean ± standard deviation. Values in B) and D) are displayed as box-plot diagrams with Tukey whiskers; + symbol marking mean. Significance levels: *** p<0.001. IgA, No.=8; albumin, No.=12.

Previous studies demonstrate PR-RBC quality similar to conventional RBCs, suggesting up to 35 days storage possibility[7,19,20]. Our results support these conclusions *in vitro*, even up to 42 days of storage.

The increased hemolysis, extracellular K^+ and RMV count in our irradiated study arm (**Figure 1E**, **F**, **I**) suggest, consistent with previous literature, that the RBC membrane is severely damaged by irradiation[25-28]. No such damage was detected after PI.

Comparison of automated washing with ACP 215 to the centrifugal washing step incorporated in the PI process demonstrated a slightly more efficient plasma removal in automated-washed RCCs (**Figures 2A-D**). However, this efficacy comes with a price: more mechanical stress, resulting in faster post-wash increase of hemolysis, extracellular K^+ and RMV count. This is expected, as mechanical stress is a well-known inducer of premature membrane deterioration[29]. European guidelines advocate total protein content <0.5 g/unit in washed RCCs[24]. We did not have access to total protein measurement, but used albumin as indirect indicator as, roughly, 50-60% of the total protein constitutes of albumin in healthy individuals[30]. As mean albumin was 0.010 g/unit in PR-RCCs, estimating 50% indicates a mean total protein content of 0.020 g/unit post-PR; 25 times below guidelines. No common European specifications exist for IgA, but when IgA reduction is the primary indication for washing, post-wash levels may still be necessary to monitor. The AABB stipulates <0.5 mg/L post-washing[31]. In our study, even though >94% reduction was achieved, none of the PR-RCCs met this criterion, whereas all automated-washed RCCs did. Such efficacy has not been reported in similar studies[22,32]. Low-volume RCCs and buffy coat removal are suggested favourable for low post-wash IgA levels[32] and could potentially explain our results. The PI centrifugation protocol was not designed to remove IgA, but amustaline

metabolites. Recently, a manual, double-centrifugation washing protocol reduced IgA more efficiently than ACP 215[22]. Possibly, similar IgA reduction may be obtained by adjusting the PI centrifugation protocol or introducing a second cycle. However, introduction of further mechanical stress may also adversely impact the RBC membrane.

Despite almost identical membrane preservation and morphology throughout time, PR-RBCs differed to reference RBCs in metabolism. Elevated ATP levels, at the expense of premature 2,3-DPG depletion (**Figure 1J**, and **N**), are likely linked to the post-PI pH reduction[33] (**Figure 1M**); an outcome of the PI process, optimised for pH dependent anchoring/metabolization of amustaline[6]. The lower pH likely haltered glucose breakdown, thus explaining the slower lactate accumulation in PR-RCCs (**Figure 1K-L**). The initially lower glucose concentration could not be linked to any negative effects on the RBCs; additionally, the excess glucose after automated washing did not implicate overall better storage outcome. 2,3-DPG is generally restored in the body within a few days post-transfusion[34,35]; however, the premature depletion (**Figure 1N**) may be of significance during trauma where reduced oxygen unloading capacity may be more critical due to the need for rapid/large-volume transfusions. This possible impact on oxygen delivery should be investigated further.

Understanding the impact of the increased ATP availability post-PI is complex. The lower generation of RMVs in PR-RCCs may be related to the ATP concentration[36]; however, other ATP-driven mechanisms such as adjustment of extracellular K^+ and RMV surface structures did not specifically favour PR-RCCs. Furthermore, irradiated RCCs, though containing equal ATP concentrations to PR and reference, diverged in percentage of RMV phosphatidylserine and CD47 (**Figure 1G-H**). Possibly, as both irradiation and PI are known to instigate oxidative stress, the two treatments may differ in the generation of reactive oxygen species (ROS) and their quenching capacity. During PI, the ROS quencher GSH is added to limit the risk of neo-antigen formation on the RBC surface[37]. The impact of GSH addition on other surface structures, as well as on the RBC metabolism[38,39], should be further explored. Overall, it might be informative to add assays for determination of markers of oxidative stress such as GSH, lipid peroxidation and total antioxidant capacity[26,40] in a future follow-up study.

The centrifugation protocol also potentially influences the RBC and RMV morphology, as RMV-CD47 expression differed between automated-washed RCCs and PR-RCCs in this study. Also, the notable post-irradiation decrease in RMV-phosphatidylserine contradicts our previous study where phosphatidylserine increased after irradiation[17]. The RMV supernatant centrifugation protocols varied between these studies. Possibly, RMV subpopulations exhibiting different surface characteristics are favoured differently by the centrifugation characteristics. Phosphatidylserine promotes phagocytosis whereas CD47 undergoes an age-related conformational change from anti-phagocytotic to pro-phagocytotic signalling in mature RBCs[41]. This could possibly explain the high initial presence of both as RMV surface ligands. The storage-dependent decrease has been previously demonstrated[36]. Its significance remains to be further explored.

Study limitations include reduced sample size for IgA (No.=8), 2,3-DPG (No.=6) and CD47 (No.=4). Processing interventions involved timeline differences: PI was executed on d2 following the manufacturer's specifications, whereas irradiation and automated washing was performed on d14 to simulate "worst-case scenario"; this must be considered when comparing washing efficacy/post-intervention storage lesion. Hemolysis and extracellular K^+ concentrations increase more rapidly when the RBCs are exposed to stress elements during late storage, when the storage lesion effects are more prominent, than during early storage[27]. Therefore, we speculate that the differences in membrane preservation properties between the study arms would likely have been smaller if irradiation and washing had been executed at the same time as PI. Furthermore, this study was conducted using blood bags plasticized with DEHP. As Europe is standing on the verge of a plasticizer exchange of large impact[16], development of a corresponding DEHP-free PI system and re-assessment of RBC quality post-PI both *in vitro* and *in vivo* in this new setting is essential. DEHP-free PI systems may also introduce the need to "upgrade" current AS such as SAGM or US equivalents AS-1/AS-5 to newer-generation AS as a

measure to mitigate hemolysis[17,18,42-44]. Obviously, a change of AS will require further validation, especially if alkali ASs are used, as the PI technique is pH sensitive. Finally, as ATP levels above approximately 2.5 µmol/g Hb correlate with >75% 24-hour survival in circulation[45], this *in vitro* study is indicative of adequate post-transfusion *in vivo* survival of PR-RBCs throughout 42 days of storage, but it does not provide solid evidence, especially as elevated post-PI ATP levels are not necessarily linked to elevated *in vivo* survival[20].

Taken together, our results suggest that RBCs treated with PI exhibit superior membrane preservation compared to both irradiated and automated-washed RCCs, proposing up to 42 days storage possibility. The metabolism is altered, but likely does not negatively impact the overall RBC quality, possibly with exception for 2,3-DPG. Consideration should be given to further development of the centrifugation protocol to optimise IgA reduction.

CONCLUSIONS

Pathogen reduced RCCs hold the potential to replace both washed and irradiated RCCs with a joint component, maintaining as long shelf-life as a conventional RCC. This could help reducing outdating rate and ensure the supply of secondary-processed RBC components. Such a replacement would be advantageous for both the blood supply and the patient safety.

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Cerus Corp. provided the Intercept Blood System.

AUTHORS' CONTRIBUTIONS

LL provided the research idea, study design, laboratory work, data analysis and manuscript composition; SO assisted on RBC laborations; TNA provided IgA analyses; EW, SL and PS provided technical and medical expertise and proof-reading; MU supervised the study. All Authors revised the manuscript.

The Authors declare no conflict of interest.

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