

Original Article

Effects of rejuvenation on young and old erythrocytes of banked blood towards the end of storage period

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Abstract: The effects of rejuvenation on the subpopulation of stored erythrocytes have not been explored. This study aims at determining the influence of rejuvenation on young and old erythrocytes of stored blood. Prior studies have shown the disappearance of young cells after day 20 of storage. Blood was stored in CPDA-1 for 35 days and erythrocytes were isolated on 25th, 30th and 35th day, revitalized using rejuvenation solution (PIPA), and separated into young and old erythrocytes using Percoll-BSA density gradient. Erythrocyte, oxidative stress and antioxidant capacity markers were assessed in the hemolysate. Young erythrocytes could be isolated beyond day 20 of storage, after rejuvenation. Antioxidant capacity of both young_{Rej} (rejuvenated young cells) and old_{Rej} (rejuvenated old cells) increased while superoxides decreased resulting in lower levels of protein oxidation & lipid peroxidation. Rejuvenation reduced storage lesion and maintained membrane sulfhydryls in both young and old erythrocytes, however, it could not restore sialic acids. Rejuvenation had more impact on young_{Rej}. A higher young: old cell ratio would be beneficial for transfusion. This study gives a comparative analysis of rejuvenation on erythrocyte aging in banked blood, thus opening new avenues towards better blood bank practices.

Keywords: Rejuvenation, young and old erythrocytes, stored blood, oxidative stress

Introduction

There is an increasing demand for blood and its components (packed red blood cells, fresh frozen plasma, cryoprecipitate and platelets), thereby a need to improve blood banking practices [1]. The transfer of the specific component (of whole blood) required by the patient is known as component therapy [2], which has formed an integral part of the treatment as it lowers the risks of post-transfusion reactions [3]. Erythrocytes are transfused to treat anemia or blood loss.

Erythrocytes have a life span of about 120 days in circulation. Alterations in physical, biochemical and physiological properties occur during aging. Thus, aged cells are more prone to be trapped and ultimately destroyed during micro-circulation [4]. Young erythrocytes have elevated cell volume, size, metabolic activity, cell surface charge, sialic acid, glucose, ATP and 2,3-DPG; while old erythrocytes have decreased

density, bending elastic modulus and mechanical fragility [5, 6].

A reducing environment is maintained in the cells of all life forms. Oxidative stress (OS) occurs when prooxidants overwhelm the antioxidant capacity [7]. Erythrocytes are prone to OS mainly due to its presence in an oxygen-rich environment and absence of nucleus and major cell organelles (no repair or macromolecule generation machinery). Oxidative insult occurs during storage, which changes the morphological, physiological, biomechanical, biochemical and metabolic properties of erythrocytes. These changes include (i) Vesiculation, membrane asymmetry and increased rigidity; (ii) Loss in ATP, 2,3-DPG, chloride and phospholipids and reduction in antioxidant capacity; and (iii) Reactive oxygen species (ROS) cascade, protein oxidation and lipid peroxidation, which reduce the efficacy of blood over long term storage [8, 9]. Erythrocytes are more susceptible to OS over storage, resulting in increased osmotic fra-

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gility, which leads to the release of cellular components into the plasma [10]. Storage lesions cause accelerated aging of erythrocytes, which are detrimental to their function and survival [4]. A reduction in the ratio of young to old cells is observed with storage. The young cells disappeared after day 20 of storage, resulting in a multitude of aged cells towards the end of the shelf life (35 days) [11, 12]. Transfusion of stored blood has shown to cause post-transfusion reactions, which is detrimental to the patient.

Attempts to restore or reverse the adverse reactions over storage have led to the development of post-storage rejuvenation treatments, such as Rejuvesol™ (Citra Labs, USA). Rejuvesol™ contains sodium pyruvate (100 mM), inosine (99.9 mM), adenine (5 mM), dibasic sodium phosphate (70.4 mM) and monobasic sodium phosphate (29 mM) having a pH of 6.7-7.4 [13]. This solution was designed to replenish the ATP pool and rectify the impaired metabolic activity. Rejuvenation of erythrocytes results in reduced adhesion to the vascular endothelium, elevation in O₂ transport [14], and a reduction in ROS [15].

Studies have shown the benefits of rejuvenation of erythrocytes (mixed population) and focused on ATP and 2,3-DPG concentrations, deformability, osmotic fragility, mechanical fragility, metabolomics, pH and *in vivo* recovery of rejuvenated erythrocytes [13-19]. Few studies have shown the disappearance of young erythrocytes beyond day 20 of storage [11, 12]. However, studies on the influence of rejuvenation on aging and storage lesion in erythrocytes have not been reported. In this study, whole blood stored beyond day 20 was rejuvenated and the impact of rejuvenation on the subpopulation of erythrocytes was assessed through oxidative stress markers. This study throws light on the continuous changes occurring after rejuvenation with storage and gives a comparative analysis of the efficacy of rejuvenated young cells vs rejuvenated old cells towards the end of the storage period (weeks 3 and 4).

Materials and methods

Reagents

Percoll (17-0891-02) was purchased from GE healthcare. Epinephrine, thiobarbituric acid

(TBA) and 4,7-diphenyl-1,10-phenanthroline disulfonic acid disodium salt were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). All other chemicals used were of reagent grade and organic solvents were of spectral grade.

Ethical approval

Ethical approval was obtained from the Kempegowda Institute of Medical Sciences Institutional Ethics Committee (KIMS-IEC).

Blood sampling

Blood was collected from healthy males aged 20-45 years, from the Kempegowda Institute of Medical Sciences (KIMS) Hospital blood bank, Bengaluru into Citrate Phosphate Dextrose Adenine-1 solution (CPDA-1).

Experimental design

Whole blood (n=16) was stored in CPDA-1 at 4°C for 35 days in polypropylene tubes. Erythrocytes were isolated on days 20, 25, 30 and 35 of storage and rejuvenated using rejuvenation solution (PIPA -a solution containing pyruvate inosine phosphate and adenine). Erythrocytes were then separated into young and old erythrocytes using a Percoll-BSA density gradient. The young and old erythrocytes obtained were used to analyze the RBC markers - hemoglobin, hemolysis and mechanical fragility. Erythrocytes were lysed and hemolysate was used to assess the following markers: superoxides, sialic acid, antioxidant defense (TAC_{CUPRAC}, Glutathione, plasma membrane redox system (PMRS), antioxidant enzymes), glucose and protein oxidation & lipid peroxidation products. The experimental design is explained in the schematic diagram (**Figure 1**).

Days 20, 25, 30 and 35 = without rejuvenation;
Days 25R, 30R and 35R = with rejuvenation;
young_{Rej} = Rejuvenated young cells; old_{Rej} = Rejuvenated old cells.

Erythrocyte separation

Erythrocytes were isolated by centrifuging whole blood at 1,000 g for 20 min at 4°C. Plasma was aspirated and the erythrocytes (pRBCs) were washed and resuspended in isotonic phosphate buffer (310 mOsm) (pH 7.4) to a final hematocrit of 50% [20].

Rejuvenation of young and old erythrocytes

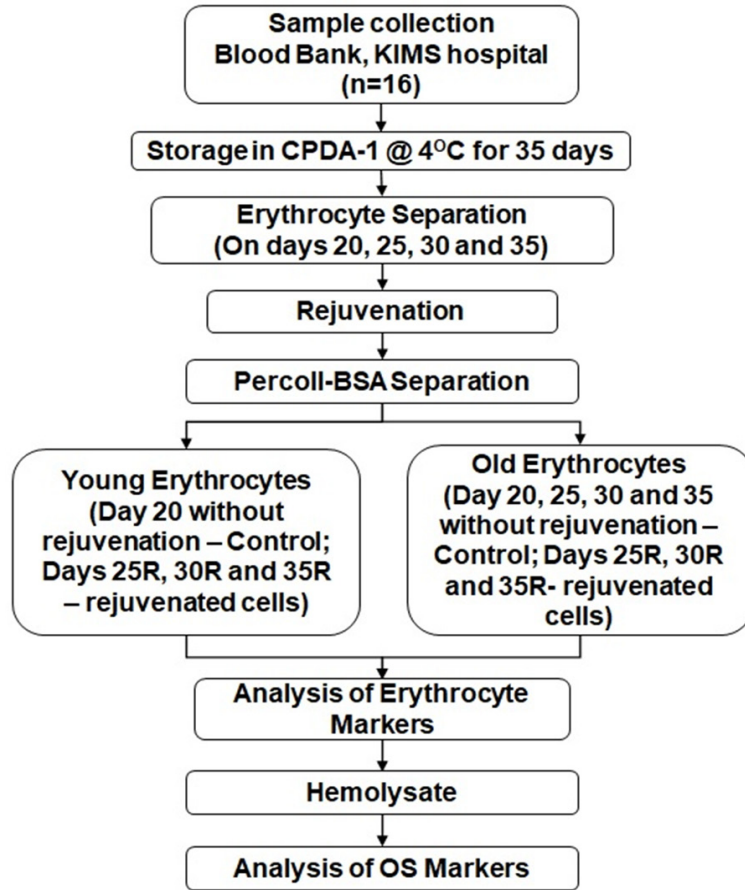


Figure 1. Experimental design.

Rejuvenation treatment

Erythrocytes isolated from stored blood were rejuvenated using rejuvenation solution (PIPA) (sodium pyruvate (100 mM), inosine (99.9 mM), adenine (5 mM), dibasic sodium phosphate (70.4 mM) and monobasic sodium phosphate (29 mM)) prepared in double distilled water and sterilized through a syringe filter (0.22 micron). The erythrocytes were incubated with PIPA at 37°C for 1 hr. The erythrocytes were washed twice using isotonic phosphate buffer [21].

Isolation of young and old erythrocytes

Young and old erythrocytes were isolated using a Percoll-BSA density gradient as described by Corsi *et al.*, 1999 [22]. Solutions of BSA were prepared, (i) 4.8% (w/v) in water and (ii) 4.8% (w/v) in Percoll. Solution A was made up of 19 parts solution (i) and 1 part HEPES buffer (pH 7.4) and solution B was made up of 19 parts solution (ii) and 1 part HEPES buffer (pH 7.4). Solutions A and B were used to form 84%, 76%

and 70% (v/v) Percoll gradients. The pRBCs isolated from whole blood were layered upon the Percoll-BSA gradient and centrifuged at 1000 rpm for 10 min at 20°C.

The cell layers at the 70% and 84% gradients (young and old erythrocytes respectively) were aspirated, washed and resuspended in isotonic phosphate buffer (pH 7.4) (hematocrit 50%). The separated young and old erythrocytes were used for further assays.

Erythrocyte markers

The cyanomethemoglobin method was used to determine the erythrocyte Hb [Hemocord reagent (Coral Clinical Systems, India)] and absorbance was measured at 540 nm [23]. Oxidative hemolysis and mechanical fragility were determined using the methods described by Şentürk *et al.*, 2001 [24] and Lippi *et al.*, 2012 [25] respectively. Oxidative hemolysis

was performed by treating erythrocytes with 0.9% PBS 1% H₂O₂ and mechanical fragility was performed by aspirating the erythrocytes twice through an insulin syringe (1 ml, 31 gauge, 0.25 × 8 mm). The amount of Hb in the supernatant was measured at 540 nm against maximum absorbance (aliquots of erythrocytes lysed with water).

Hemolysate was prepared using hypotonic phosphate buffer (20 imOsm) to lyse erythrocytes, which was stored at -20°C for further assays.

Sialic acids were estimated using the method described by Warren, 1959 [26]. Hemolysate was treated with arsenite solution and 0.6% TBA (in 0.5 M sodium phosphate) and incubated in a boiling water bath for 15 min. Cyclohexanone was added and centrifuged at 1000 rpm for 5 min. The absorbance of the supernatant was read at 549 nm and the amount of sialic acid was calculated using the molar extinction coefficient for N-acetylneuraminic acid: 57,000 M⁻¹cm⁻¹.

Rejuvenation of young and old erythrocytes

Metabolic markers

Glucose was estimated by the enzymatic GOD-POD method (Autospan Gold kit) [27]. In the presence of glucose oxidase, glucose gets converted into gluconic acid and H_2O_2 . The latter reacts with p-hydroxybenzoic acid and 4 amino-antipirine forming a red quinoneimine dye. The intensity (measured at 546 nm) is proportional to the amount of glucose present. Lactate dehydrogenase (LDH) was estimated using the method described by Buhl and Jackson, 1978 [28]. LDH reagent [reagents 1 (80 mM Tris, 1.6 mM Pyruvate and 200 mM NaCl) and reagent 2 (0.2 mM NADH) in the ratio 4:1] was added to hemolysate and incubated at 37°C for 5 min and the absorbance was read at 340 nm.

Antioxidant defenses

The method described by Beutler *et al.*, 1963 [29] was used to estimate glutathione. Hemolysate was treated with 4% sulphosalicylic acid and centrifuged at 2500 g for 15 min. 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (10 mM) was added to the supernatant and absorbance was measured at 412 nm.

The total antioxidant capacity: cupric ion reducing antioxidant capacity assay (TAC_{CUPRAC}) was measured using the method of Da Cruz, 2003 [30]. Hemolysate was treated with 0.25 mM BCS in 10 mM phosphate buffer (pH 7.4) and an initial reading was measured at 490 nm. $CuSO_4$ (0.5 mM) was added and incubated for 3 min at RT. To arrest the reaction, Ethylenediaminetetraacetic acid (EDTA) (0.01 M) was added and the final reading was measured at 490 nm. The results were expressed against a standard uric acid curve and represented in mM uric acid equivalents/l.

The method of Avron and Shavit, 1963 [31] was used to determine the erythrocyte trans-plasma membrane redox activity (PMRS). The PMRS activity is measured by following the reduction of ferricyanide. The amount of ferrocyanide was measured at 535 nm using 4,7-diphenyl-1,10-phenanthrolinedisulfonic acid disodium salt. The results are expressed in $\mu\text{mol ferrocyanide/ml hemolysate}/30 \text{ min}$ ($e = 20,500 \text{ M}^{-1}\text{cm}^{-1}$).

Antioxidant enzyme activities of superoxide dismutase (SOD) [EC 1.15.1.1] and catalase

(CAT) [EC 1.11.1.6] were determined using the method described by Misra and Fridovich, 1972 [32] and Aebi 1984 [33] respectively. SOD was measured spectrophotometrically at 480 nm and expressed as the amount of enzyme that inhibits 50% oxidation of epinephrine. CAT activity was measured after treating hemolysate with absolute alcohol and incubated at 0°C. H_2O_2 (6.6 mM) and phosphate buffer were added and the reduction in absorbance was measured at 240 nm ($e = 43.6 \text{ M}^{-1}\text{cm}^{-1}$).

Oxidative stress markers

The markers of oxidative stress were determined through superoxide levels [34]; lipid peroxidation products - Conjugated Dienes [34] and malondialdehyde (MDA) [35]; and protein oxidation products - advanced oxidation protein products (AOPP) [36] and protein sulfhydryls (SH) [37].

Superoxides were measured by the reduction of Cytochrome C, measured at 550 nm and the results were expressed as $\mu\text{M}/\text{mg protein}$.

Conjugated dienes were measured by treating hemolysate with ether: ethanol [1:3 (v/v)]. The amount of conjugated dienes was measured in the supernatant at 235 nm.

MDA was measured in the hemolysate, after treatment with SDS (8.1%), acetic acid (20%) and thiobarbituric acid (TBA) (0.6%) and incubated in a boiling water bath. The reaction mixture was cooled and a mixture of butanol-pyridine (15:1) was added and centrifuged at 1000 rpm for 5 min. The absorbance of the supernatant was measured at 532 nm using the standard, 1,1,3,3-tetramethoxy propane and represented as $\mu\text{mol}/\text{mg protein}$.

AOPP was measured by treating hemolysate with isotonic phosphate buffer, 1.16 Ml^{-1} potassium iodide and glacial acetic acid. The absorbance of the reaction mixture was read immediately at 340 nm and estimated using the extinction coefficient of $26 \text{ M}^{-1}\text{cm}^{-1}$.

SH was measured by treating hemolysate with sodium phosphate buffer (0.08 Ml^{-1}) with Na_2 -EDTA (0.5 mg/ml) and SDS (2%). 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (20 mg in 10 ml). The absorbance was measured at 412 nm and

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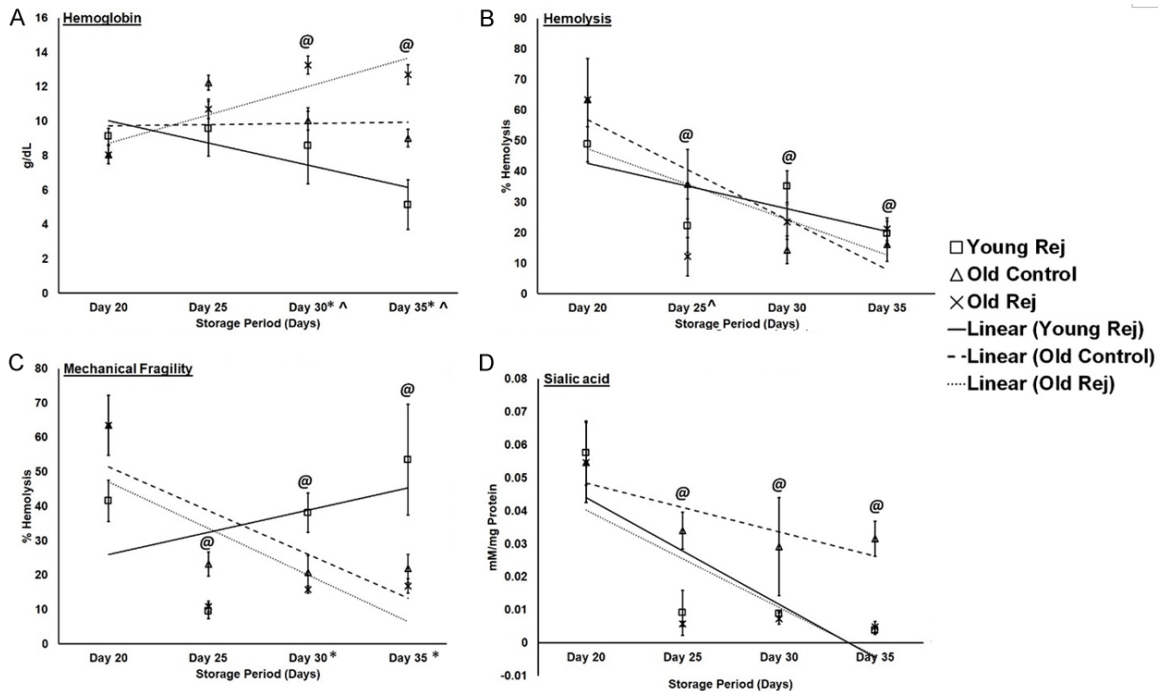


Figure 2. Erythrocyte Markers in Rejuvenated Young and Old Erythrocytes of Stored Blood. (A) Hemoglobin; (B) Hemolysis; (C) Mechanical Fragility; and (D) Sialic Acid. Values are represented as Mean \pm SE and linear trendlines for each group are plotted. # - Young_{Rej} significant against Day 20 (Controls); @ - Old_{Rej} significant against Day 20 (Controls). Particular storage days: * - Young_{Rej} significant with Old_{Rej}; ^ - Old_{Rej} significant with Old cells (controls). Changes in hemoglobin ($P < 0.0001$), hemolysis ($P < 0.01$) and sialic acid ($P < 0.001$) were significant with storage in Old_{Rej}. The variations between old_{Rej} and controls were significant in hemoglobin ($P < 0.0001$) and hemolysis ($P < 0.01$). Variations between young_{Rej} and old_{Rej} were significant in hemoglobin ($P < 0.0001$) and mechanical fragility ($P < 0.0001$).

determined using molar absorptivity, 13,600 $M^{-1}L^{-1}cm^{-1}$.

Protein determination

The protein concentration of the hemolysate was determined using Lowry's method against a bovine serum albumin (BSA) standard [38].

Statistical analyses

Results of Day 20 of young cells vs young_{Rej} (days 25R, 30R and 35R); and day 20 of old cells vs old_{Rej} (days 25R, 30R and 35R) were analyzed using one-way ANOVA, followed by Bonferroni Post-test. The results of old cells (days 25, 30 and 35) vs old_{Rej} (days 25R, 30R and 35R); and young_{Rej} vs old_{Rej} on days 25R, 30R and 35R were analyzed using two-way ANOVA, followed by Bonferroni Post-test.

ANOVA and Bonferroni Post-test were performed using GraphPad Prism 6 software and $P < 0.05$ was considered significant. The results

are expressed as Mean \pm SE and linear trendlines are plotted.

Results

Young erythrocytes could not be isolated beyond day 20 of storage in controls as cells in the 70% Percoll-BSA disappeared. However, after rejuvenation, young cells could be isolated on days 25, 30 and 35.

Erythrocyte markers

Hb: Changes in Hb were significant in old_{Rej} ($P < 0.0001$) with storage. Hb increased by 60% on days 30R and 35R against day 20 in old_{Rej}.

Rejuvenation increased Hb in old_{Rej} on days 30R and 35R against controls ($P < 0.0001$).

Hb increased on days 30R (55%) and 35R (1 fold) in old_{Rej} when compared with young_{Rej} ($P < 0.0001$) (Figure 2A).

Rejuvenation of young and old erythrocytes

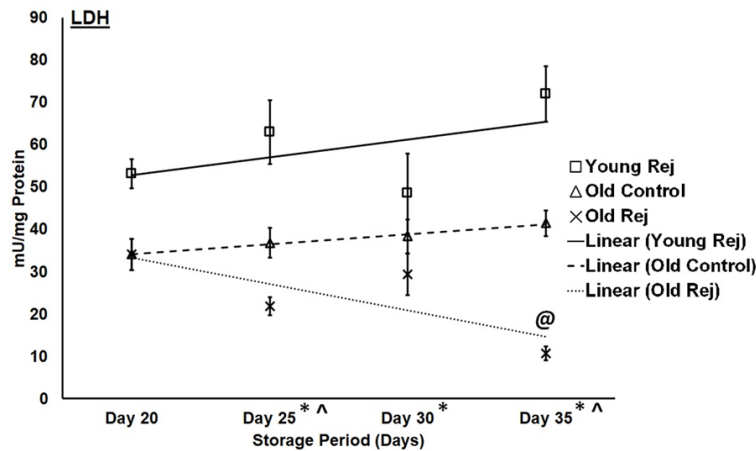


Figure 3. Lactate Dehydrogenase (LDH) in Rejuvenated Young and Old Erythrocytes of Stored Blood. Values are represented as Mean \pm SE and linear trendlines for each group are plotted. # - Young_{Rej} significant against Day 20 (Controls); @ - Old_{Rej} significant against Day 20 (Controls). Particular storage days: * - Young_{Rej} significant with Old_{Rej}; ^ - Old_{Rej} significant with Old cells (controls). Changes in LDH were significant with storage in old_{Rej} ($P < 0.0001$), between old_{Rej} & controls ($P < 0.0001$) and between young_{Rej} & old_{Rej} ($P < 0.0001$).

Hemolysis: Hemolysis reduced significantly in old_{Rej} by 82% (day 25R) and 75% (days 30R and 35R) with day 20 ($P < 0.01$).

Hemolysis reduced on day 25R (66%) in old_{Rej} against controls ($P < 0.01$).

Changes in hemolysis were insignificant between young_{Rej} and old_{Rej} (**Figure 2B**).

Mechanical fragility: Mechanical fragility decreased significantly in old_{Rej} by 80% on days 25R, 30R and 35R against day 20.

Changes in mechanical fragility were insignificant between old cells and old_{Rej}.

Mechanical fragility decreased by 65% on days 30R and 35R in old_{Rej} when compared with young_{Rej} ($P < 0.0001$) (**Figure 2C**).

Sialic acid: Significant changes were observed in old_{Rej} ($P < 0.001$) with storage. Sialic acid in old_{Rej} reduced by 1 fold on days 25R, 30R and 35R when compared with day 20.

Young_{Rej} and old_{Rej} did not show any variations with their respective controls (**Figure 2D**).

Metabolic markers

Glucose: Glucose was significant in old_{Rej} ($P < 0.0001$) with storage, where elevations of 1

fold were observed on days 30R and 35R with day 20.

Variations in glucose were insignificant between old cells and old_{Rej}; and between young_{Rej} and old_{Rej}.

LDH: Changes in LDH were significant in old_{Rej} ($P < 0.0001$) with storage, where a decrement of 70% was observed on day 35R against day 20.

Old_{Rej} had lower LDH on days 25R and 35R (75%) when compared with controls ($P < 0.0001$).

LDH decreased in old_{Rej} on days 25R (65%), 30R and 35R (85%) with respect to young_{Rej} ($P < 0.0001$) (**Figure 3**).

Antioxidant defenses

TAC_{CUPRAC}: Changes in TAC_{CUPRAC} were insignificant with storage.

Rejuvenation reduced TAC_{CUPRAC} in old_{Rej} on days 25R (50%), 30R and 35R against days 25, 30 and 35 respectively ($P < 0.0001$).

TAC_{CUPRAC} was lower on day 35R in old_{Rej} when compared with young_{Rej} ($P < 0.01$) (**Figure 4A**).

PMRS: Significant changes were observed in young_{Rej} during storage ($P < 0.0001$). PMRS elevated in young_{Rej} on days 25R, 30R (55%) and 35R (77%) when compared with day 20.

PMRS increased by 65% in old_{Rej} on days 25R and 30R against controls ($P < 0.0001$).

An elevation of 40% was observed in young_{Rej} on days 25R and 35R with old_{Rej} ($P < 0.0001$) (**Figure 4B**).

Glutathione: Changes in glutathione were significant in old_{Rej} during storage ($P < 0.0001$), where decrements of 1 fold were observed in old_{Rej} on days 25R, 30R and 35R with day 20.

Glutathione reduced by 1 fold in old_{Rej} on days 30R and 35R when compared with controls ($P < 0.0001$).

Rejuvenation of young and old erythrocytes

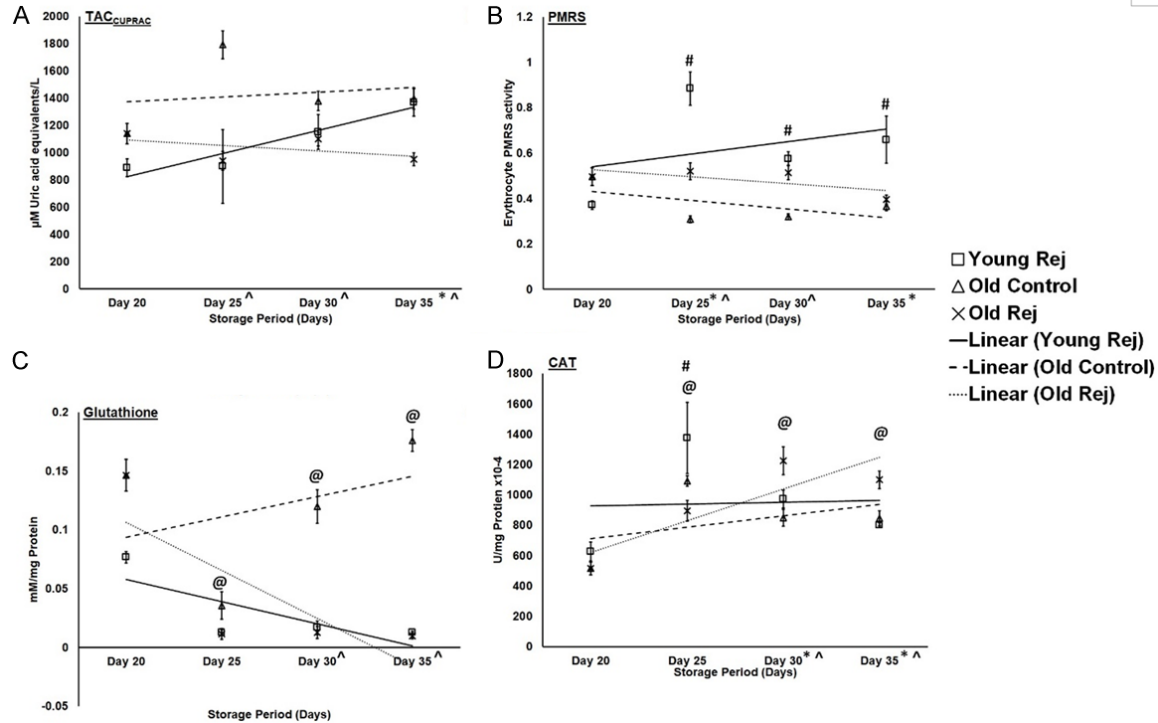


Figure 4. Antioxidant Defense in Rejuvenated Young and Old Erythrocytes of Stored Blood. (A) Total antioxidant capacity: Cupric Ion Reducing Antioxidant Capacity Assay ($\text{TAC}_{\text{CUPRAC}}$); (B) Plasma Membrane Redox System (PMRS); (C) Glutathione and (D) Catalase (CAT). Values are represented as Mean \pm SE and linear trendlines for each group are plotted. # - Young_{Rej} significant against Day 20 (Controls); @ - Old_{Rej} significant against Day 20 (Controls). Particular storage days: * - Young_{Rej} significant with Old_{Rej}; ^ - Old_{Rej} significant with Old cells (controls). Changes in PMRS ($P < 0.0001$) & CAT ($P < 0.0001$) were significant with storage in young_{Rej} and glutathione ($P < 0.0001$) & CAT ($P < 0.0001$) with storage in old_{Rej}. The variations between old_{Rej} and controls were significant in $\text{TAC}_{\text{CUPRAC}}$ ($P < 0.0001$), PMRS ($P < 0.0001$), glutathione ($P < 0.0001$) and catalase ($P < 0.0001$). Variations between young_{Rej} and old_{Rej} were significant in $\text{TAC}_{\text{CUPRAC}}$ ($P < 0.01$), PMRS ($P < 0.0001$) and catalase ($P < 0.001$).

Variations in glutathione were insignificant between young_{Rej} and old_{Rej} (**Figure 4C**).

SOD: Rejuvenation did not have any significant effect on both young and old cells.

CAT: CAT was significant in young_{Rej} ($P < 0.0001$) and old_{Rej} ($P < 0.0001$) with storage. Catalase was higher in young_{Rej} on day 25R when compared with day 20. Rejuvenation increased CAT by 74% (day 25R) and 1 fold (days 30R and 35R) in old_{Rej} when compared with day 20.

Rejuvenation increased CAT activity on days 30R and 35R in old_{Rej} with controls ($P < 0.0001$).

CAT activity was also higher in old_{Rej} when compared with young_{Rej} on days 30R and 35R ($P < 0.001$) (**Figure 4D**).

Oxidative stress markers

Superoxides: Superoxides were similar throughout storage in both young and old cells.

A reduction in superoxide was observed in old_{Rej} on day 35R against controls ($P < 0.01$).

Changes between young_{Rej} and old_{Rej} were insignificant (**Figure 5A**).

Conjugated dienes: Changes in conjugated dienes were significant with storage. Conjugated dienes reduced by 60% on days 30R and 35R against day 20 in young_{Rej} ($P < 0.0001$). Reductions in conjugated dienes were observed in old_{Rej} on days 25R (60%), 30R (67%) and 35R (77%) when compared with day 20 ($P < 0.0001$).

Rejuvenation reduced conjugated dienes by 50% in old_{Rej} on days 25R, 30R and 35R with their respective controls ($P < 0.0001$).

Conjugated dienes elevated by 45% on day 25R in old_{Rej} against young_{Rej} ($P < 0.0001$) (**Figure 5B**).

Rejuvenation of young and old erythrocytes

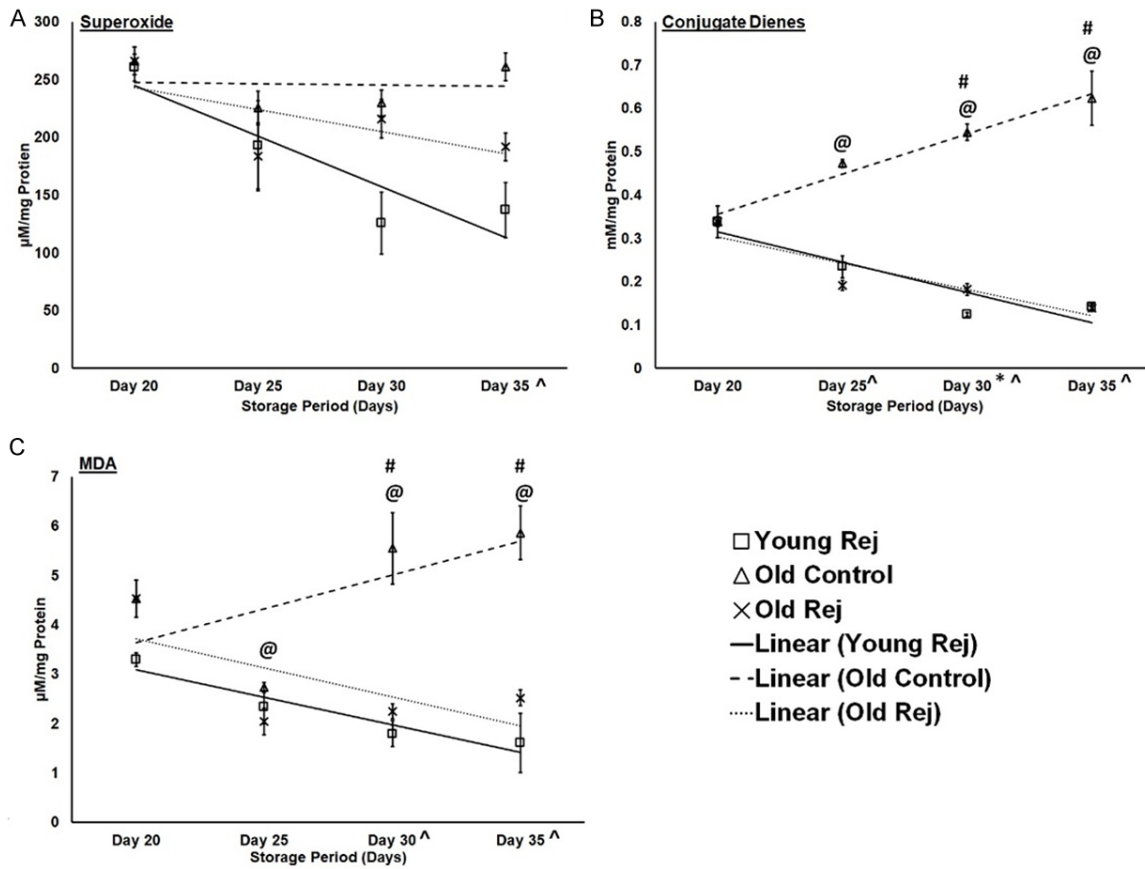


Figure 5. Oxidative Stress Markers in Rejuvenated Young and Old Erythrocytes of Stored Blood. (A) Superoxides; (B) Conjugated Dienes; and (C) Malondialdehyde (MDA). Values are represented as Mean \pm SE and linear trendlines for each group are plotted. # - Young_{Rej} significant against Day 20 (Controls); @ - Old_{Rej} significant against Day 20 (Controls). Particular storage days: * - Young_{Rej} significant with Old_{Rej}; ^ - Old_{Rej} significant with Old cells (controls). Changes in conjugate dienes ($P < 0.0001$) and MDA ($P < 0.0001$) were significant with storage in young_{Rej} and old_{Rej}. The variations between old_{Rej} and controls were significant in superoxides ($P < 0.01$), conjugate dienes ($P < 0.0001$) and MDA ($P < 0.0001$). Variations between young_{Rej} and old_{Rej} were significant in conjugate dienes ($P < 0.0001$).

MDA: MDA in young_{Rej} ($P < 0.0001$) and old_{Rej} ($P < 0.0001$) were significant during storage. MDA reduced on days 30R and 35R in young_{Rej}, whereas on days 25R, 30R and 35R in old_{Rej} by 50% against day 20.

Rejuvenation lowered MDA in old_{Rej} by 60% on days 30R and 35R when compared with their respective controls ($P < 0.0001$).

Variations were insignificant between young_{Rej} and old_{Rej} (**Figure 5C**).

AOPP: Changes in AOPP were significant with storage in both young_{Rej} ($P < 0.0001$) and old_{Rej} ($P < 0.0001$). Rejuvenation reduced AOPP in both young and old cells on days 25R, 30R and 35R with day 20.

AOPP in old cells decreased on days 25R and 35R when compared with controls ($P < 0.0001$).

AOPP was higher on days 30R and 35R (73%) in old_{Rej} against young_{Rej} ($P < 0.001$) (**Figure 6A**).

SH: Changes in SH were insignificant with rejuvenation over storage.

SH increased by 1 fold (day 30R) and 70% (day 35R) in old_{Rej} against controls ($P < 0.0001$) (**Figure 6B**).

Discussion

Previous studies have focused on the effects of rejuvenation on a mixed population of erythrocytes. However, this study attempts to compare the effects of rejuvenation on (i) young

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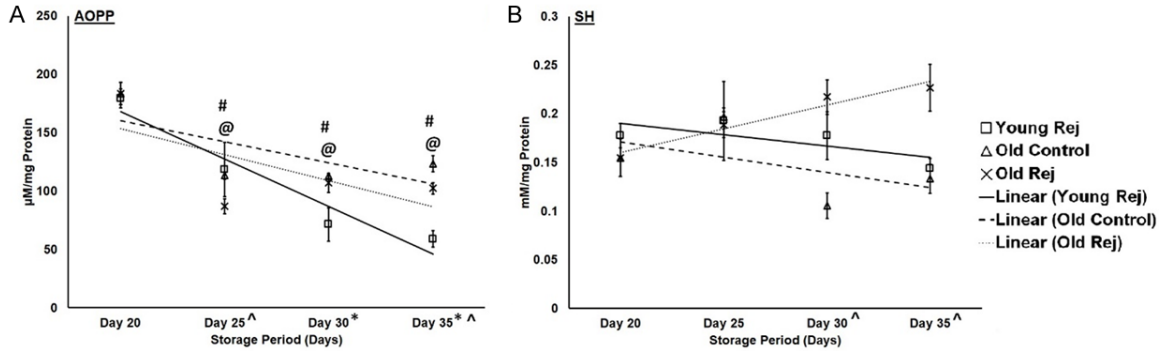


Figure 6. Protein Oxidation Products in Rejuvenated Young and Old Erythrocytes of Stored Blood. (A) Advanced Oxidation Protein Products (AOPP); and (B) Sulfhydryls (SH). Values are represented as Mean \pm SE and linear trendlines for each group are plotted. # - Young_{Rej} significant against Day 20 (Controls); @ - Old_{Rej} significant against Day 20 (Controls). Particular storage days: * - Young_{Rej} significant with Old_{Rej}; ^ - Old_{Rej} significant with Old cells (controls). Changes in AOPP ($P < 0.0001$) were significant with storage in young_{Rej} and old_{Rej}. The variations between old_{Rej} and controls were significant in AOPP ($P < 0.0001$) and SH ($P < 0.0001$). Variations between young_{Rej} and old_{Rej} were significant in AOPP ($P < 0.001$).

cells and (ii) old cells during storage; and (iii) young_{Rej} vs old_{Rej}.

Percoll-BSA fraction (70%) of young cells disappeared beyond day 20 of storage [11, 12]. However, rejuvenating stored erythrocytes yielded young erythrocytes even after day 20, till the end of storage. Rejuvenation revitalizes the erythrocytes by reducing the Ca²⁺ levels, phosphatidylserine (PS) exposure, and increasing their deformability [13, 15, 18]. Rejuvenation restores the glycolytic flux of the erythrocytes [39], thereby increasing the metabolic activity and ATP levels [13, 17]. This was evident in the elevated LDH levels of young_{Rej}. Rejuvenation reduces reactive oxygen species (ROS) [16] and this was in concurrence with the results of superoxides, TAC_{CUPRAC}, sulfhydryls and antioxidant enzymes (SOD and CAT) in young_{Rej}. Decrements in superoxides can be correlated with TAC_{CUPRAC} ($r = -0.799$), protein oxidation (AOPP) ($r = 0.986$) and lipid peroxidation products - conjugated dienes ($r = 1$) and MDA ($r = 0.981$). Rejuvenation can restore most of the changes caused due to storage lesion, however, few changes are irreversible [13]. This was substantiated in the results of glutathione, which reduced over storage.

Elevations in Hb of old_{Rej} can be attributed to the increase in the number of young cells getting converted into old ones with storage. Hb increments can also be due to the decrease in vesiculation, reduction in PS exposure and elevated deformability after rejuvenation [13,

15, 18]. This reduced mechanical fragility and increased sulfhydryls, however, hemolysis was maintained throughout storage. Decrements in superoxides can be attributed to lower levels of ROS after rejuvenation [15]. This was evident in the results of superoxides which correlated with SOD ($r = 0.99$), PMRS and catalase. Reduction in superoxides (OS) also correlated with decrease in protein oxidation (AOPP) ($r = 0.972$) and lipid peroxidation products - conjugated dienes ($r = 0.907$) and MDA ($r = 0.926$). Thereby total antioxidant capacity (TAC_{CUPRAC}) was maintained during storage. LDH decreased in old_{Rej} with storage, as old erythrocytes have a lower metabolic activity than young cells [40].

Hemoglobin in young_{Rej} was lower than old_{Rej}, which can be attributed to the decrease in the number of young cells due to the increase in mechanical fragility in young_{Rej}. Young erythrocytes may have endured extensive damage that was irreversible through rejuvenation after day 20. The antioxidant status of young_{Rej} improved in comparison to old_{Rej}. Rejuvenation lowered ROS levels more efficiently in young_{Rej} as antioxidant enzyme activity reduces with age [11]. This was evident in the results of TAC_{CUPRAC}, PMRS and Catalase. Increments of LDH in young_{Rej} confirmed the reports of Xue and Yeung [40], that young erythrocytes are metabolically more active than old erythrocytes.

Rejuvenation was beneficial in negating the effects of storage to a certain extent and helped

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to reduce the overall OS in both young and old erythrocytes. The antioxidant capacity of both young_{Rej} and old_{Rej} increased after rejuvenation, however, young_{Rej} had a higher antioxidant capacity and lower protein & lipid peroxidation products. Rejuvenation significantly reduced storage lesion in both young and old erythrocytes, but could not completely restore sialic acid levels.

The main highlight of this study is that young erythrocytes could be isolated from whole blood beyond day 20 of storage after rejuvenation till day 35 (shelf life of banked blood in CPDA-1). Therefore, a higher young: old cell ratio would help in reducing the storage lesion as young cells are more resilient to OS and rejuvenation had a more pronounced effect on them. Higher fractions of young cells would also be beneficial as they remain in circulation for a longer period than old cells, post-transfusion. This study accentuates the prospects of rejuvenation in improving blood bank practices.

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Disclosure of conflict of interest

None.

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