

The Expression of CD47 and its Association with Other Biochemical Storage

Lesions in Stored Non-leucoreduced versus Leucoreduced Red Blood Cell Units

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ABSTRACT

Background: CD47 is an integrin associated protein which decreases with PRBCs (packed red blood cells) prolonged storage that possibly leads to less effective blood transfusion. Electrolyte imbalance occurs due to inactivation of Na⁺/K⁺ ATPase pump in low temperature which in turn leads to continuous increase of extracellular potassium, which is considered life threatening for patients with renal failure or those with massive transfusions. The objective of the present study is to evaluate the expression of CD47 by flow cytometry with other biochemical storage changes (pH, sodium and potassium ions) in CPD/SAGM packed non-leucoreduced Group (A) versus CPD/SAGM leucoreduced Group (B) red blood cell units at different storage times (on day 7, 14, 28 from date of red blood cells collection).

Subjects and Methods: Nearly 450 ml of whole blood is obtained into quadruple blood bags and further subdivided into 20 non-leucoreduced units and 20 leucoreduced units. Non-leucoreduced and leucoreduced units are stored at 4 ± 2°C. Samples are taken from each packed red blood cell unit at days 7, 14 and 28 and tested for Na⁺ and K⁺ ions, pH and CD47 marker by flow cytometry.

Results: Both leucoreduced and non-leucoreduced units show a highly significant decrease in Na⁺, pH and CD47 expression and a highly significant increase in K⁺ level. However, there was no statistically significant difference found between the 2 studied groups regarding Na⁺ and K⁺ levels, pH and CD47 expression on packed red cells except for Na⁺ on 28th day which was slightly higher in leucoreduced than non-leucoreduced units.

Conclusion: Red cell storage lesion is an inevitable process. Our study showed a highly statistically significant decrease in CD47 by flow cytometry in both leucoreduced and non-leucoreduced PRBCs along the storage period from day 7 to day 28.

Keywords: CD47, Biochemical Storage Lesions, Stored Non-leucoreduced, Red Blood Cell Units, Sodium, Potassium, Ain Shams University.

INTRODUCTION

Blood transfusion saves thousands of lives every day. It is mandatory in cases of acute bleeding as in accidents or in surgeries ⁽¹⁾.

This emergent need for blood required blood storage at low temperature which induced biochemical and biomechanical changes, collectively known as RCSL (red cell storage lesion) ⁽²⁾.

Electrolyte imbalance occurs due to inactivation of Na⁺/K⁺ ATPase pump in low temperature which in turn leads to continuous increase of extracellular K⁺ which is considered life threatening for patients with renal failure or those with massive transfusions ⁽³⁾. Spontaneous oxidation of hemoglobin to methemoglobin is catabolized into iron. Free iron reacts with other free radicals causing membrane protein and lipid oxidation with subsequent red cell membrane damage ⁽⁴⁾.

Microvesicles increase on red cell storage; it is a subset of protein and phospholipids, laden with hemoglobin in addition to externalization of phosphatidylserine that increases risk of thrombosis ⁽⁵⁾.

Activated and apoptotic leucocytes in stored PRBCs could trigger storage lesions, since they represent a source for bioactive factors like oxygen free radicals, cytokines and enzymes. Therefore,

leucoreduction exhibits a beneficial effect on RBCs storage lesion by improving both the hemolysis and post-transfusion recovery of leucoreduced RBCs ⁽⁶⁾.

CD47 initially named integrin associated protein is a cell surface protein of the immunoglobulin superfamily. It was demonstrated by **Eldesouky et al.** ⁽⁷⁾ as well as many other studies that the expression of CD47 on red blood cells decreases with its prolonged storage which leads to a decrease in “don’t eat me” signals sent to macrophages and this may consequently triggers RBCs clearance from the recipient’s circulation which probably means less effective blood transfusion.

The aim of our study was to evaluate the expression of CD47 by flow cytometry in addition to other biochemical storage changes (pH, Sodium and potassium ions) in CPD/SAGM packed non-leucoreduced versus CPD/SAGM leucoreduced red blood cell units at different storage times (on day 7, 14, 28 from date of red blood cells collection).

SUBJECTS AND METHODS

1. Sample preparation

This study was conducted at the central blood bank of Ain Shams University Hospitals. Forty donors (100% males) who met the standards of blood donation criteria for allogenic blood transfusion [WHO guidelines for

blood donor selection ⁽⁸⁾] from January 2021 to October 2021 were enrolled into the study.

The forty units are divided into two groups according to their leucoreduction status; Group (A) for non-leucoreduced units and Group (B) for leucoreduced units, where whole blood of 20 units only was filtered through (spacell RZ-2000) filter with residual leucocytes $<1 \times 10^6$ which keeps residual leucocytes averaging below 1×10^6 . Filtration was done within 12 hours from blood donation.

All whole blood units from both groups are then centrifuged using SIGMA 8KS centrifuge for 15 minutes at 4°C at a speed of 4000 rpm/min to separate plasma yielding non-leucoreduced and leucoreduced packed red blood cell units. Non-Leucoreduced and leucoreduced units are stored at $4 \pm 2^\circ\text{C}$.

Samples are taken from each packed red blood cell unit at days 7, 14 and 28 from date of whole blood unit collection and tested for: sodium (Na⁺), potassium (K⁺), pH, CD47 marker by flow cytometry.

2. Sodium, Potassium and pH

Sodium and potassium measurement was carried out on Beckmen coulter chemistry analyzer (AU480). pH was measured using blood gases analyzer (GEM 3500).

3. Flow cytometric analysis of CD47

RBCs were analyzed by NAVIOS 2 Laser 6 Color FCM [Beckman Coulter, USA] using PE (Phycoerythrin) conjugated Anti-CD47 monoclonal antibody (Clone B6H12(RUO); BD Pharmingen™).

A gate for RBCs population which excluded debris and agglutinates was set by forward versus side scatter in logarithmic scale. Cells expressing CD47 were fluorescently stained with the intensity of staining directly proportional to the density of CD47 expression on RBCs. The positivity was expressed as a percentage with a cut-off of $>20\%$ over the corresponding isotypic control in addition to mean fluorescence intensity (M.F.I).

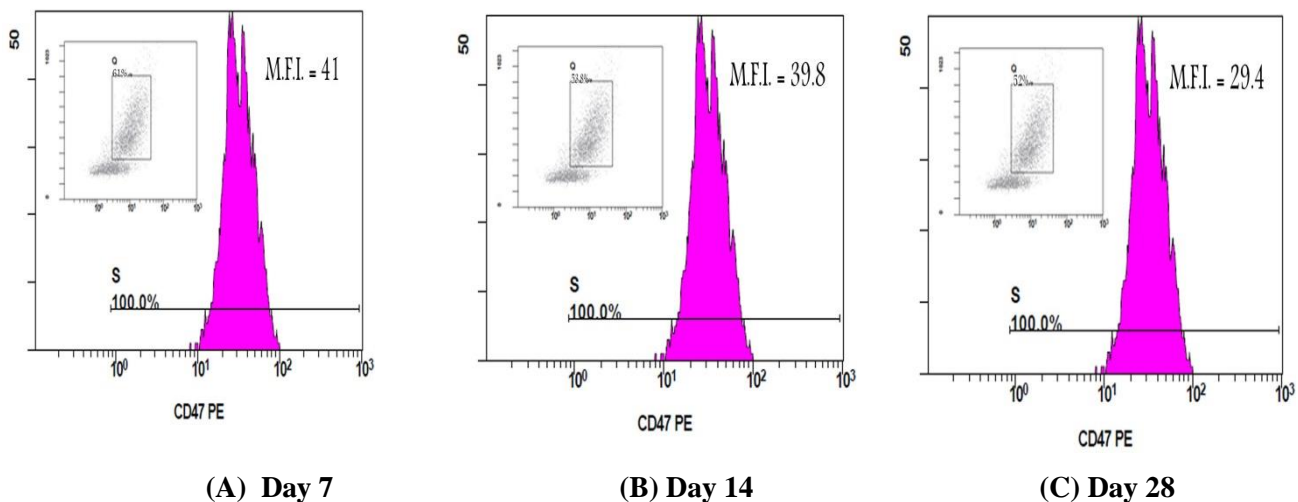


Figure (1): Representing the gradual decrease in flow cytometric expression of CD47 (%/M.F.I) from our study along the storage periods day 7(A), day 14(B), day 28(C).

Ethical consent:

An approval of the study was obtained from Ain Shams University Academic and Ethical Committee. Every patient signed an informed written consent for acceptance of participation in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical Analysis:

Data were collected, revised, coded and entered to the Statistical Package for Social Sciences (IBM SPSS) version 23. The quantitative variables were presented as mean, standard deviations (SD) and range, while qualitative variables were presented as numbers and

percentages. The p-value was considered significant as the following: P-value >0.05: Non-significant (NS), P-value <0.05: Significant (S), P-value <0.01: Highly significant (HS).

RESULTS

As regard the comparison between non-leucoreduced (Group A) leucoreduced (Group B) units at days 7, 14 and 28; There was no statistically significant difference between the two studied groups regarding sodium and potassium levels, pH and CD47 expression (%/M.F.I) except for sodium at day 28 which was slightly higher in Group (B) than in Group (A) with mean value of 144.9 mEq/L in leucoreduced units and 137.67 mEq/L in non-leucoreduced units (Table 1).

Table (1): Comparison between Non-leucoreduced and Leucoreduced regarding their biochemical parameters and CD47 expression at days 7, 14, and 28.

| Storage days | Parameters measured | Non-leucoreduced (Group A) | Leucoreduced (Group B) | P-value |
|-------------------------------|---------------------|----------------------------|------------------------|------------|
| Day 7 (Mean ± SD) | Sodium (mEq/L) | 149.85 ± 3.79 | 151.1 ± 4.92 | 0.374 (NS) |
| | Potassium (mEq/L) | 9.06 ± 2.66 | 8.53 ± 1.74 | 0.611 (NS) |
| | pH | 6.97 ± 0.07 | 6.94 ± 0.07 | 0.124 (NS) |
| | CD47 (%) | 60.38 ± 4.7 | 59.48 ± 5.84 | 0.592 (NS) |
| | CD47 (M.F.I) | 42.35 ± 5.78 | 43.1 ± 9.24 | 0.760 (NS) |
| Day 14 (Mean ± SD) | Sodium (mEq/L) | 146.7 ± 5.81 | 148.25 ± 4.66 | 0.358 (NS) |
| | Potassium (mEq/L) | 15.56 ± 3.61 | 13.71 ± 3.87 | 0.146 (NS) |
| | pH | 6.84 ± 0.07 | 6.85 ± 0.07 | 0.585 (NS) |
| | CD47 (%) | 56.34 ± 4.13 | 52.59 ± 7.71 | 0.063 (NS) |
| | CD47 (M.F.I) | 41.54 ± 5.78 | 39.44 ± 5.86 | 0.262 (NS) |
| Day 28 (Mean ± SD) | Sodium (mEq/L) | 137.67 ± 8.54 | 144.9 ± 8.71 | 0.012 (S) |
| | Potassium (mEq/L) | 23.76 ± 4.81 | 20.63 ± 4.82 | 0.053 (NS) |
| | pH | 6.73 ± 0.07 | 6.72 ± 0.06 | 0.622 (NS) |
| | CD47 (%) | 49.19 ± 8.1 | 50.68 ± 12.33 | 0.673 (NS) |
| | CD47 (M.F.I) | 35.77 ± 3.7 | 35.05 ± 7.72 | 0.707 (NS) |

P>0.05: Non-significant (NS); P <0.05: Significant (S); P <0.01: Highly significant (HS).

In non-leucoreduced blood units (Group A); there was gradual decrease in sodium level, pH, CD47 expression (%/M.F.I) at the 3 different assay times. Sodium levels showed a highly significant decrease from 149.85 at day 7 to 146.7 at day 14 to reach 137.67 mEq/L at day 28 (P-value <0.001). Also, pH level showed a highly significant decrease from 6.97 to 6.84 at day 7, 14 respectively to reach 6.73 at day 28 (P-value <0.001). The CD47 expression (%/M.F.I) showed a highly significant decrease with baseline 60.38% / 42.35 at day 7 which decrease at day 14 to 56.34% / 41.54 and reaches finally 49.19% / 35.77 at day 28 (P-value <0.001). In contrast, potassium level showed a highly significant increase subsequently with different assay time; its mean level at day 7 was 9.06 mEq/L, at day 14 was 15.56 and at day 28 was 23.76 (P-value <0.001) (Table 2).

Table (2): Follow up for the biochemical changes and CD47 expression at day 7, 14, and 28 among non-leucoreduced blood units (Group A).

| Non-leuco-reduced Group (A) | Day 7 | Day 14 | Day 28 | P-value |
|------------------------------|---------------|---------------|---------------|-------------|
| | No. = 20 | No. = 20 | No. = 20 | |
| Sodium (mEq/L) (Mean ±SD) | 149.85 ± 3.79 | 146.70 ± 5.81 | 137.67 ± 8.54 | <0.001 (HS) |
| Potassium (mEq/L) (Mean ±SD) | 9.06 ± 2.66 | 15.56 ± 3.00 | 23.76 ± 4.81 | <0.001 (HS) |
| pH (Mean ±SD) | 6.97 ± 0.07 | 6.84 ± 0.07 | 6.73 ± 0.07 | <0.001 (HS) |
| CD47 (%) (Mean ±SD) | 60.38 ± 4.70 | 56.34 ± 4.13 | 49.19 ± 8.10 | <0.001 (HS) |
| M.F.I (Mean ±SD) | 42.35 ± 5.78 | 41.54 ± 5.78 | 35.77 ± 3.70 | 0.001 (HS) |

P>0.05: Non-significant (NS); P <0.05: Significant (S); P <0.01: Highly significant (HS).

In leucoreduced blood units (Group B); there was gradual decrease in sodium level, pH, CD47 expression (% / M.F.I) at the 3 different assay times. Sodium level showed a significant decrease from 151.1 at day 7 to 148.25 at day 14 to reach 144.9 mEq/L at day 28 (P-value <0.05). pH level showed a highly significant decrease from 6.94 to 6.85 at day 7, 14 respectively to reach 6.72 at day 28 (P-value <0.001). The CD47 expression (%/M.F.I) shows a significant decrease with baseline at day 7 was 59.48% / 43.1 which decrease at day 14 to 52.59% / 39.44 and reaches finally 50.68% / 35.05 at day 28 with P-value <0.05 for CD47% and P-value <0.001 for M.F.I. In contrast, Potassium level showed highly significant increase (P-value <0.001) with different assay times; its mean level

at day 7 was 8.53 mEq/L, at day 14 was 13.71 and 20.63 at day 28 (Table 3).

Table (3): Follow up for the biochemical changes and CD47 expression at day 7, 14, and 28 among leucoreduced blood units (Group B).

| Leuco-reduced Group (B) | Day 7 | Day 14 | Day 28 | P-value |
|-----------------------------|---------------|---------------|---------------|-------------|
| | No. = 20 | No. = 20 | No. = 20 | |
| Sodium (mEq/L) (Mean±SD) | 151.10 ± 4.92 | 148.25 ± 4.66 | 144.90 ± 8.71 | 0.013 (S) |
| Potassium (mEq/L) (Mean±SD) | 8.53 ± 2.11 | 13.71 ± 3.21 | 20.63 ± 5.11 | <0.001 (HS) |
| pH (Mean±SD) | 6.94 ± 0.07 | 6.85 ± 0.07 | 6.72 ± 0.06 | <0.001 (HS) |
| CD47 (%) (Mean±SD) | 59.48 ± 5.84 | 52.59 ± 7.71 | 50.68 ± 11.33 | 0.022 (S) |
| M.F.I (Mean±SD) | 43.10 ± 9.24 | 39.44 ± 5.86 | 35.05 ± 7.72 | <0.001 (HS) |

P>0.05: Non-significant (NS); P <0.05: Significant (S); P <0.01: Highly significant (HS).

A statistically significant positive correlation was found between CD47 expression (%/ M.F.I), sodium and pH level on all stored PRBC units throughout its storage period from day 7 to day 28 with P-values (0.011/ 0.000) / (0.007/ 0.000). A statistically significant negative correlation was found between CD47 expression (%/M.F.I) and potassium (P-value 0.012/ 0.000) (Table 4).

Table (4): Correlation between biochemical parameters and CD47 expression on all stored PRBC units throughout its storage period from day 7 to day 28.

| Variable | CD47 (%) | | M.F.I | |
|-----------|----------------|----------|-----------------|---------|
| | r | P- value | R | P-value |
| Sodium | 0.232* | 0.011 | -0.243** | 0.007 |
| Potassium | -0.228* | 0.012 | -0.333** | 0.000 |
| pH | 0.352** | 0.000 | -0.383** | 0.000 |

DISCUSSION

Prolonged cold storage of PRBCs inhibits Na⁺/K⁺ pump which lead to continuous leakage of intracellular potassium and progressive decrease of extracellular sodium. Also, lactic acid accumulation leads to lowering pH. Although, it has been postulated that Na⁺/K⁺ pump activity is restored in vivo 1-4 days post transfusion, hyperkalemia in arrhythmic patients, patients with renal failure and those with massive transfusion is still a possible sequelae ⁽³⁾.

CD47 is the only known five transmembrane (TM) receptor of the immune system that has many roles in signal transduction, cellular proliferation and

apoptosis through its binding to its ligands. Recently, CD47 was additionally considered as anti-phagocytic marker on RBCs which sends 'don't eat me' signal to macrophages through binding to signal regulatory protein (SIRP- α)⁽⁹⁾.

It was demonstrated by many studies that the expression of CD47 on red blood cells decreases with prolonged storage of RBCs which leads to a decrease in anti-phagocytic signals which eventually leads to a decrease in the 24-h in vivo post transfusion recovery of RBCs and this may decrease the effectiveness of blood transfusion.

The aim of our study is to evaluate the expression of CD47 by flow cytometry with other biochemical storage changes (pH, Sodium and potassium ions) in CPD/SAGM packed non-leucoreduced Group (A) versus CPD/SAGM leucoreduced Group (B) red blood cell units at different storage times (on day 7, 14, 28 from date of red blood cells collection).

Our results showed a highly statistically significant decrease in CD47 expression along the storage period with M.F.I values of 42.35 at day 7, 41.54 at day 14 and 35.77 at day 28 in Group A Versus 43.10 at day 7, 39.44 at day 14 and 35.05 at day 28 in Group B.

Our results were consistent with **Eldesouky et al.**⁽⁷⁾ who studied CD47 expression and 2,3 DPG in 100 leucoreduced and non-leucoreduced PRBCs and revealed a progressive significant decrease in CD47 M.F.I on day 0, 1, 21 and recommended further studies on RCSL along days of 7, 14 and 28 post donation which has been implemented in our study. Also, our results were close to those of **Kamel et al.**⁽¹⁰⁾ since, the decrease in CD47 expression on day 1, 14 and 28 was statistically significant in 43 bags of non-leucoreduced and buffy coat removed PRBCs.

On the contrary, **Farahat and Sharaf**⁽¹¹⁾ observed a non-significant decrease in CD47 expression during all storage times from day 1 to day 42 (P-value 0.196).

Regarding biochemical lesions, there was a strong positive correlation between storage duration of PRBCs and biochemical changes (Na, K and pH) in both leucoreduced and non-leucoreduced units. Sodium and pH showed highly statistically significant decrease along the storage period. Unlike, potassium that showed highly statistically significant increase from day 7 to day 28.

Our results were in accordance with **Oyet et al.**⁽³⁾ who advocate that whole blood stored for >14 days has reduced efficacy with increased markers of red cell storage lesion such as increased potassium level, lactate and fall in pH.

Minimizing the concentration of WBCs in transfused blood through leucoreduction prevented many blood transfusion adverse effects and also improves both hemolysis and post transfusion recovery of RBCs. However, Our study found that there was no statistically significant difference between non-

leucoreduced units Group (A) and leuco-reduced units Group (B) regarding biochemical parameters (Na, K & pH) and CD47 expression at day 7, 14, 28 except for Sodium at day 28 which was slightly higher in Group (B) than Group (A). Comparable results were elicited by **Shastri et al.**⁽¹²⁾ who found no statistically significant difference between buffy-coat reduced and non-buffy-coat reduced CPD/SAGM bags in biochemical parameters. Also, **Kamel et al.**⁽¹⁰⁾ also observed no effect on plasma hemoglobin and potassium levels in buffy coat reduced units.

On the contrary, **Saini et al.**⁽¹³⁾ found that leucoreduced units expressed statistically significant lower level of potassium than non-leucoreduced units. Also, **Kamel et al.**⁽¹⁰⁾ and **Eldesouky et al.**⁽⁷⁾ have found a statistically significant difference between leucoreduced and non-leucoreduced units in terms of CD47 expression throughout the storage period. However, **Kamel et al.**⁽¹⁰⁾ didn't find any significant difference regarding potassium.

CONCLUSION

Our study showed a highly statistically significant decrease in CD47 by flow cytometry in both leucoreduced and non-leucoreduced PRBCs along the storage period from day 7 to day 28. Red cell concentrates show significant decrease in sodium and pH and increase in the level of potassium during storage. Unlike many other studies, leucoreduction didn't affect the expression of neither CD47 nor biochemical parameters (Na, K and pH) from day 7 to day 28 of PRBC storage.

Conflict of interest: The authors declare no conflict of interest.

Sources of funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contribution: Authors contributed equally in the study.

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