Heliyon 10 (2024) e32056

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Evaluation of stored red blood cell quality after washing using immune indices

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ARTICLE INFO

Keywords:
Washed red blood cells
CD47
Phosphatidylserine
Blood transfusion
Physiological–biochemical parameters

ABSTRACT

Washed red blood cells (RBCs) can be used to treat immune-related diseases. However, whether the washing process changes the quality of RBCs and affects the curative effect of transfusion therapy remains unclear. We retrospectively analysed the clinical data of patients who received blood transfusion. The physiological and biochemical parameters of RBCs were tested on an automated haematology-biochemical analyser. CD47 and phosphatidylserine (PS) plasma membrane expression were analysed using flow cytometry. Morphological changes in RBCs were observed using scanning electron microscopy. The results showed that the curative effect on patients who received washed RBCs was weaker than that on those who received non-washed RBCs. Physiological and biochemical parameters of RBCs were not significantly different. RBC immune indices changed significantly after washing. The expression of "don't eat me" signals was weakened, whereas the intensity of "eat me" signals was enhanced. This study suggests that the current use of physiological and biochemical parameters as indicators to evaluate the quality of RBCs may not be comprehensive and that evaluation of the real status of RBCs requires other effective parameters. Immune molecules in RBCs are expected to become supplementary markers for evaluating RBC quality.

1. Introduction

Transfusion of stored red blood cells (RBCs) is considered an important method for saving lives during clinical transfusion treatments [1]. The removal of over 98 % of whole blood plasma from washed red blood cells, a component of stored blood products, is thought to effectively remove allergens such as plasma proteins and irregular antibody components, as well as RBC metabolites such as potassium (K^+) and lactic acid. This, in turn, is thought to effectively reduce adverse transfusion reactions including allergic reactions and non-haemolytic fever [2,3]. RBC washing is widely used in the transfusion treatment of patients with plasma component allergies, immunoglobulin A (IgA) deficiency, non-homogenous haematopoietic stem cell transplants, hyperkalaemia, or liver and kidney dysfunction [4].

The immune function of RBCs is particularly important for the regulation of the immune microenvironment [5]. Energy

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https://doi.org/10.1016/j.heliyon.2024.e32056
Received 7 February 2023; Received in revised form 28 May 2024; Accepted 28 May 2024
Available online 28 May 2024

consumption, metabolite accumulation, morphological changes in cell membranes, downregulated expression of immune molecules, and other phenomena during RBC storage are considered forms of storage damage [6,7]. Stored RBCs may be associated with a series of adverse transfusion reactions, such as clinical mortality, recurrence, and infection [8].

Washing is a secondary process for stored blood, during which the stored RBCs are centrifuged, and the maintenance solution is changed, which may cause damage to the stored RBCs [9]. Physiological and biochemical parameters are often used as indicators of RBCs quality [10]. A previous study reported no alterations in the physiological and biochemical parameters of washed RBCs, indicating that their quality was reliable [11]. However, little consideration has been given to whether RBC washing exacerbates the degree of damage to stored RBCs, including changes in immune function, and the effectiveness of RBC transfusion.

To evaluate more accurately the quality of washed RBCs, in addition to assessing physiological and biochemical parameters, the immune status of RBCs should be considered. This study focused on the effect of transfusion of washed RBCs and detected changes in related immune indicators of washed RBCs, such as CD47 and phosphatidylserine (PS), to further explore whether immune indicators can be used as supplementary indicators to evaluate the quality of RBCs.

2. Materials and methods

2.1. Data collection

We excluded patients with factors that might affect the effectiveness of RBC transfusion, including active bleeding, positive irregular antibodies, high fever, and splenomegaly. The data of 223 patients with cross-matching blood transfusion were collected from medical records at Jinling Hospital.

2.2. Preparation of RBCs

This study was conducted at the Blood Bank Unit of Jinling Hospital. Whole blood (400 ml) donated by voluntary donors was collected and 12 whole-blood samples stored for 14 days were selected to prepare RBCs; of these, 6 samples were used to prepare the washed RBCs. Whole blood was collected using disposable leukocyte filter blood bags produced by Fresenius Kabi Medical Supplies Co., Ltd (Guangzhou, China), batch No. 85SM25FA00.

2.3. Preparation of washed RBCs

The washed RBCs were prepared by professionals. A sterile connection machine provided by Wuhan BMS Medicaltech Co., Ltd. (Wuhan, China) was used to connect the RBC bag to a washing liquid bag manufactured by Shandong Weigo Group Medical Polymer Products Co., Ltd. (Weihai, China), batch No. 2107061. A 0.9 % sodium chloride solution was injected into the RBC bag and mixed slowly and gently. Centrifugal force was set at 4827 *g*, the mixture was centrifuged at 4 °C for 10 min, and the supernatant was then removed. The procedure was repeated three times, followed by addition of 100 ml RBC preservation solution to the RBC bag.

2.4. Detection of physiological and biochemical parameters in RBCs

RBC count, haemoglobin (Hb), haematocrit (Hct), mean RBC volume (MCV), and RBC distribution width (RDW) were determined using an automated haematology analyser (Sysmex XE-5000; Wakinohama-Kaigandori, Japan). Sodium (Na^+), Potassium (K^+), and glucose concentrations were measured using an automated biochemical analyser (Hitachi 7600; Tokyo, Japan). Glycated Hb was measured using an automatic meter (Bio-Rad D-10; CA, USA). Blood samples were cultured for bacteria using the BD BACTEC™ automated blood culture system (Becton Dickinson, New Jersey, USA).

2.5. Analysis of CD47 and PS plasma membrane expression using flow cytometry

Some of the collected RBCs were washed twice with phosphate-buffered saline (PBS; Gibco, USA) and resuspended at a final concentration of 10^6 cells/mL. All samples were processed within 4 h. Labelled tubes were prepared and 5 μL of the RBC preparation was added, followed by 5 μL of the appropriate (PE)-conjugated CD47 antibody (Clone No. CC2C6D4). The contents in the test tube were then mixed and incubated at 4 °C for 30 min in the dark. The cells were resuspended using 2 mL PBS, centrifuged at 300 *g* for 10 min, and the supernatant was discarded. Considering that Annexin V is translocated to the outer membrane surface during the early stages of apoptosis along with PS, Annexin V is often used to reflect the actual condition of PS. In this study, 500 μL 1 × Annexin V Binding buffer was added to resuspend the cells, followed by addition of 5 μL fluorescein isothiocyanate (FITC)-conjugated Annexin V antibody. The contents were mixed in the test tube and incubated at room temperature for 15 min in the dark. The incubated samples were immediately tested using a BD CantoII flow cytometer (BD Biosciences, Franklin Lakes, NJ).

2.5.1. Imaging of RBCs using scanning electron microscopy (SEM)

SEM analyses of RBCs were performed using an electron microscope (Hitachi SU8100; Tokyo, Japan). We randomly selected 5 blood samples from each group. The samples were fixed in phosphate-buffered (pH 7.4) 2.5 % glutaraldehyde for 2 h, washed three times in 0.1 m phosphate buffer (pH 7.4) for 15 min, post-fixed in 1 % osmium tetroxide for 1 h, washed three times in 0.1 m phosphate buffer (pH 7.4) for 15 min, and dehydrated in graded ethanol solutions (30, 50, 70, 80, 90, 95, 100, and 100 %) for 15 min. The samples

were dried in a critical point dryer, covered with a gold-palladium layer (Hitachi MC1000; Tokyo, Japan), and subjected to SEM analysis. The morphology of 200–300 cells was observed in randomly selected fields.

2.6. Statistical analysis

All statistical analyses were performed using the GraphPad Prism software 9.4.1 (GraphPad Software, San Diego, CA, USA). Frequency distributions determined the percent of samples. The data were expressed as the mean ± SD. The means and standard error of the means (SEM) between two groups were assessed using Student's t-test. The correlation between rates of clinical status was analysed using the chi-square test. Variables with a p value of less than 0.05 were considered statistically significant.

3. Results

3.1. Patients' demographic characteristics

A total of 223 patients were screened for the study, of whom 111 received transfusions of washed RBCs and 112 of non-washed RBCs. The average age was 47.96 ± 17.27 and 54.39 ± 16.24 years for the recipients of washed and non-washed RBCs, respectively. The patients were transfused 2.53 ± 1.38 and 2.42 ± 0.66 U of washed and non-washed RBCs, respectively; the difference was not statistically significant. In addition, our results showed that gender, ABO blood type, and disease status were independent of the type of transfused RBCs (Table 1).

3.2. Comparison of blood transfusion effect of different RBCs

To evaluate the effect of blood transfusion of washed and non-washed RBCs, we examined physiological parameters of RBCs in the patients. Our results showed significant increases in RBC counts, Hb concentration and HCT after blood transfusion with washed and non-washed RBCs ($p < 0.001$). Other indicators, such as MCHC, MCV and RDW did not differ significantly before and after transfusion with washed or non-washed RBCs (Table 2).

We further evaluated potential differences in the effect of blood transfusion with washed and non-washed RBCs. We compared Hb concentration and the RBC count between the two groups and found no difference among the patients before transfusion (Fig. 1A and B). However, after transfusion, Hb concentration and RBC counts were higher in the patients who received non-washed RBCs ($p < 0.001$) (Fig. 1A and B). In addition, we measured changes in Hb concentration and RBC counts in patients after blood transfusion with each unit of RBCs. Similarly, patients who received non-washed RBCs had significantly higher mean Hb concentration and RBC counts than patients who received washed RBCs ($p < 0.01$) (Fig. 1C and D).

3.3. Physiological and biochemical parameters in RBCs

The quality of the RBCs was confirmed by evaluating physiological and biochemical parameters. Our results showed that washing did not cause significant changes in the RBC counts, HCT, Hb, MCHC, MCV, or RDW (Table 3). Moreover, extracellular K^+ and serum glucose levels were significantly reduced (Fig. 2A and B), while extracellular Na^+ concentrations significantly increased, in the washed RBCs (Fig. 2C). Washing did not affect the glycosylated Hb levels in the RBCs (Fig. 2D).

Table 1
Patients' demographic characteristics distribution for transfusions of washed and non-washed RBCs.

	Washed RBCs	Non-washed RBCs	χ^2	p
Number of RBCs (U), Mean ± SD	2.53 ± 1.38	2.42 ± 0.66		0.247
Number of patients, n	111	112		
Age (year), Mean ± SD	47.96 ± 17.27	51.73 ± 14.74		0.090
Gender, n (%)				
Male	47 (42.34)	57 (50.89)	1.638	0.200
Female	64 (57.66)	55 (49.11)		
ABO Blood Groups, n (%)				
O Blood Group	31 (36.94)	24 (21.43)	1.267	0.260
A Blood Group	43 (29.73)	42 (37.50)	3.432	0.180
B Blood Group	31 (27.93)	38 (33.93)	0.939	0.332
AB Blood Group	6 (5.40)	8 (7.14)	0.286	0.593
Diseases, n (%)				
kidney diseases	33 (29.73)	31 (27.68)	0.115	0.735
autoimmune diseases	29 (26.13)	23 (20.54)	0.974	0.324
traumatic injury	15 (13.51)	16 (14.28)	0.028	0.868
neoplasms	13 (11.71)	15 (13.39)	0.144	0.705
inflammatory diseases	21 (18.92)	27 (24.11)	0.888	0.346

Table 2
The analysis of the effect of blood transfusion on washed RBCs and non-washed RBCs.

	Washed RBCs			Non-washed RBCs		
	Before blood transfusion (24 h)	After blood transfusion (24 h)	*p* value	Before blood transfusion (24 h)	After blood transfusion (24 h)	*p* value
RBC counts (n, 10^{12})	2.16 ± 0.62	2.57 ± 0.57	<0.001	2.32 ± 0.426	2.83 ± 0.44	<0.001
HCT[a] (%)	0.199 ± 0.045	0.243 ± 0.052	<0.001	0.223 ± 0.128	0.254 ± 0.032	<0.05
Hb[a] (g/L)	64.50 ± 14.93	77.19 ± 14.52	<0.001	68.02 ± 9.50	83.70 ± 10.70	<0.001
MCHC[a] (g/L)	324.24 ± 24.25	329.12 ± 15.96	0.113	321.66 ± 16.95	326.50 ± 29.85	0.099
MCV[a] (fL)	94.55 ± 11.77	92.25 ± 8.53	0.134	92.13 ± 8.23	90.73 ± 7.05	0.137
RDW[a] (%)	17.50 ± 3.61	17.95 ± 3.69	0.408	16.67 ± 2.83	16.83 ± 2.52	0.613

[a] Hematocrit, HCT; hemoglobin, Hb; Mean corpuscular hemoglobin concentration, MCHC; Mean corpuscular volume, MCV; Red cell distribution width, RDW.

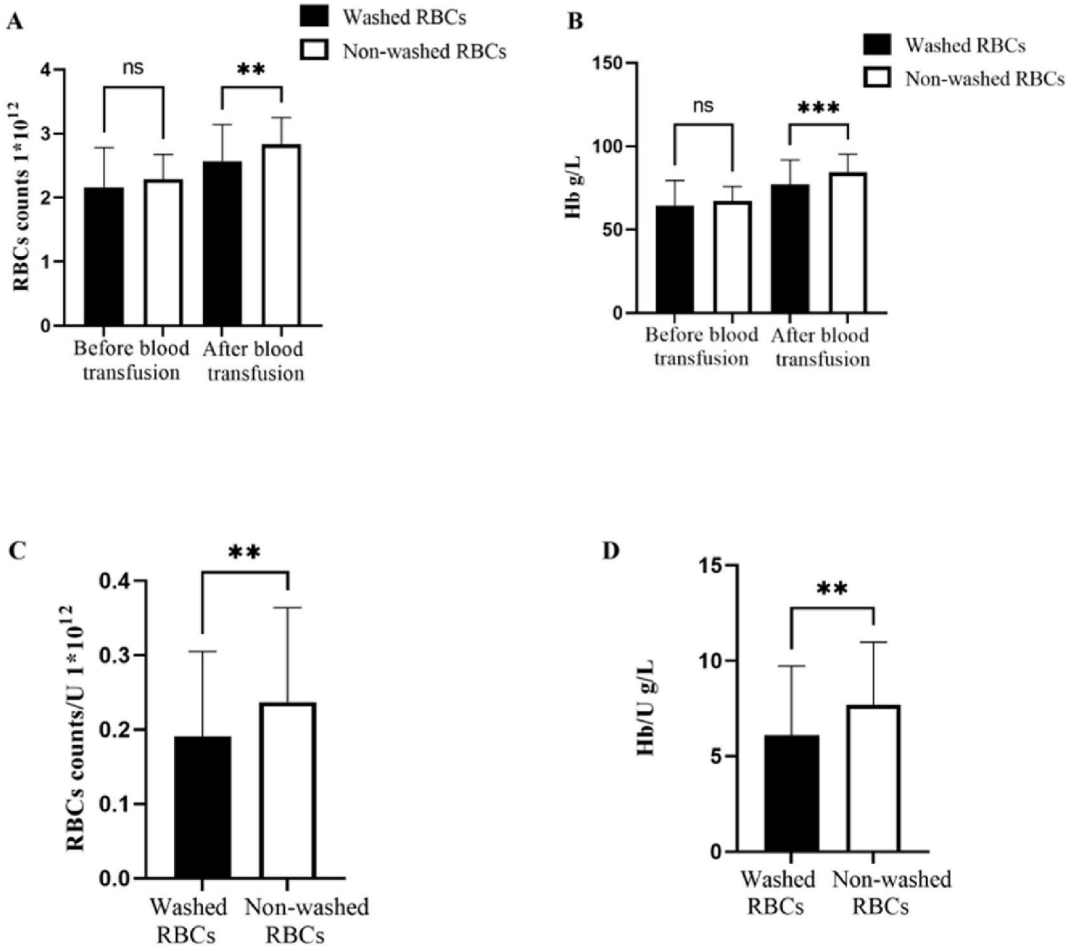

Fig. 1. Changes in Hb values and RBCs counts during blood transfusion. (**A,B**) Comparison of Hb values and RBCs counts before and after transfusion of washed and non-washed RBCs within 24 h; (**C,D**) The mean increase in the number of Hb and RBCs per unit of blood transfusion. $^{**}p<0.01$, $^{***}p<0.001$, $^{ns}\ p > 0.05$.

3.4. Evaluation of CD47 and PS plasma membrane expression using flow cytometry

RBCs stored for 14 days were selected to detect the levels of CD47 and PS exposed on the plasma membranes using flow cytometry. Washing did not affect CD47 expression in the whole RBCs ($p > 0.05$), but remarkably decreased the levels of CD47 proteins in non-apoptotic stored RBCs from 94.28 ± 2.58 % to 86.70 ± 1.82 % ($p < 0.0001$). In contrast, significantly increased expression of PS, from 5.72 ± 2.89 % to 13.43 ± 1.93 %, was observed in the washed RBCs that were stored for 14 days ($p < 0.001$) (Fig. 3A and B). To further

Table 3
Effects of washing on physiological parameters in RBCs.

	Non-washed RBCs	Washed RBCs	*p* value
RBCs counts (n, 10^{12})	5.94 ± 0.34	5.76 ± 0.21	0.172
HCT[a] (%)	0.51 ± 0.02	0.49 ± 0.01	0.066
Hb[a] (g/L)	169.10 ± 8.13	164.30 ± 5.77	0.145
MCHC[a] (g/L)	306 ± 2.16	300 ± 3.74	0.121
MCV[a] (fL)	95.57 ± 3.59	96.87 ± 2.68	0.640
RDW[a] (%)	15.70 ± 0.62	15.68 ± 0.65	0.815
bacterial culture	No bacterial growth found	No bacterial growth found	

[a] Hematocrit, HCT; hemoglobin, Hb; Mean corpuscular hemoglobin concentration, MCHC; Mean corpuscular volume, MCV; Red cell distribution width, RDW.

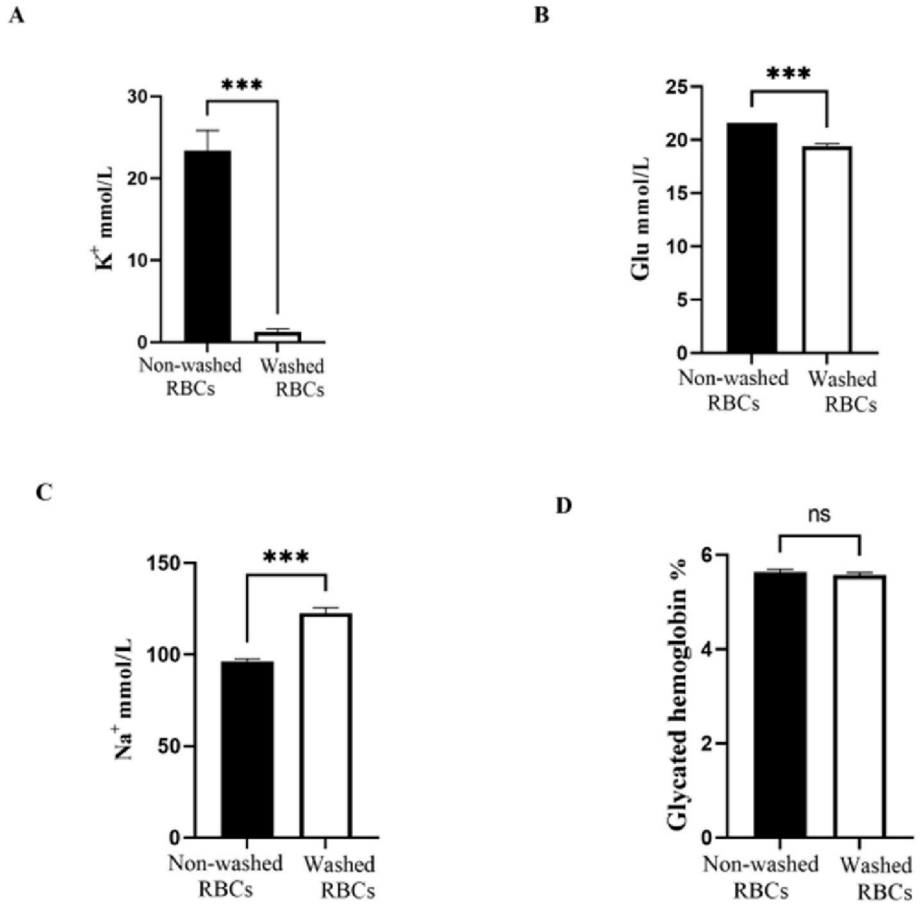

Fig. 2. Effects of washing on biochemical parameters in RBCs. Changes in extracellular K^+ (**A**), serum glucose (**B**), extracellular Na^+ (**C**) and glycated hemoglobin (**D**) in washed RBCs and non-washed RBCs. ***$p<0.001$, ^{ns} $p > 0.05$.

evaluate the sustained effect of washing on RBC membrane proteins, we measured the expression of CD47 and PS at 21, 28, and 35 days. Our results showed that significant changes in CD47 expression in the non-apoptotic washed RBCs was noted from day 14 of storage ($p < 0.001$). Similarly, the expression of PS was significantly different with the extension of storage time ($p < 0.001$), suggesting that the washing process could aggravate the occurrence of apoptosis (Fig. 3A,B,C,D).

3.5. Morphological changes in RBCs observed using SEM

Differences between the two groups of RBCs were evident in the SEM images. As storage duration increased, the cells started to transform into spherocytes and echinocytes on day 14 of storage (Fig. 4A). Washing aggravated the formation of spherocytes and echinocytes (Fig. 4B). Further changes in the RBC membranes were observed after washing; specifically, a higher degree of echinocytosis was noted at high magnification (10,000×). This change could worsen the formation of microvesicles (Fig. 4C and D).

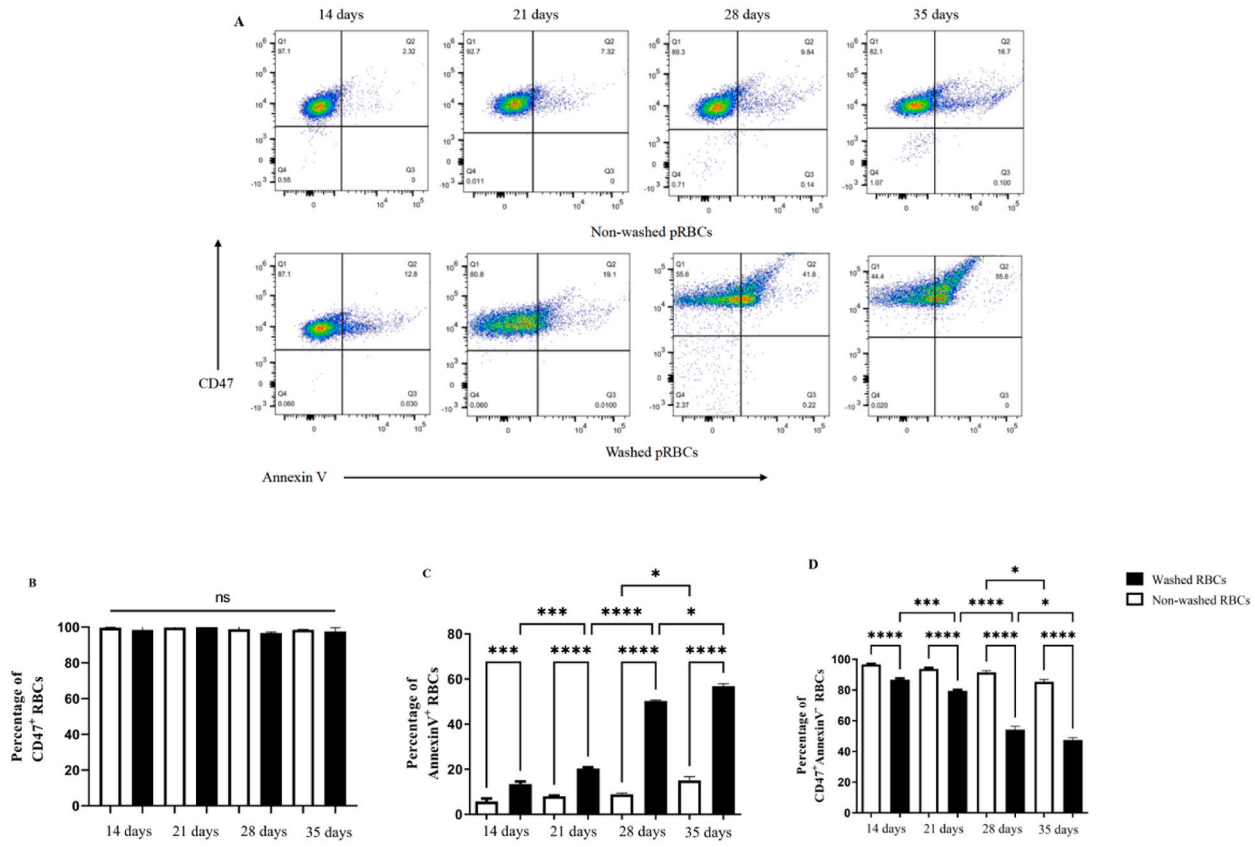

Fig. 3. The results of CD47 and PS Plasma Membrane Expression by Flow Cytometry. (**A,B,C**) The stored RBCs were divided into 14 days, 21 days, 28 days and 35 days, and the expression of CD47 and PS in each group were detected immediately at the set time point. (**D**) The expression of CD47 in non-apoptotic RBCs. **p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

4. Discussion

Blood transfusion therapy is a vital component of modern clinical healthcare and RBCs are among the most commonly used blood components [12]. The primary RBC preparations are additive-solution RBCs, leukocyte-depleted red cell suspensions, packed RBCs, and washed RBCs [13–15]. RBC washing uses normal saline to remove plasma proteins, irregular antibody components, and metabolites. It is suitable for treating patients with IgA deficiency or those who have had a transfusion reaction [16]. Washed RBCs are used to reduce the supernatant K^+ load in RBCs and prevent the development of hyperkalaemia [17]. Both manual and automated methods are used to prepare washed RBCs. Studies have shown that manual washing results in better RBC quality, increased removal of plasma proteins, reduced haemoglobin loss, and more reliable cell quality [9]. Therefore, manual washing of RBCs was adopted in this study to reduce RBC damage. However, as metabolites accumulate during storage, RBC quality deteriorates, eventually impairing RBC function, a condition known as a storage lesion [18]. Storage damage typically includes changes in RBC pathophysiology, such as altered RBC metabolism, increased red cell oxidative stress, RBC membrane damage, and reduced red cell viability. The efficacy of transfusion with storage-lesioned RBCs remains controversial, with studies indicating that transfusion of stored RBCs is associated with higher rates of infection, mortality, and recurrence, as well as lower cure rates [19]. Therefore, RBC quality is related to the safety of clinical transfusion therapy.

Previous studies have used physiological and biochemical parameters of RBCs to evaluate their quality. Some investigations found no alterations in physiological or biochemical parameters during washing [11]. Washing was assumed not to harm the RBCs because they could generally perform their physiological and biochemical functions. The washed RBCs were therefore considered reliable. RBCs are known to play a role in cytokine signalling and immune cell activity modulation [20]. The immune status of RBCs is closely related to their quality. As a result, the parameters used to assess RBC quality should be fully determined and alterations in the immunological regulatory function of RBCs should be investigated further. This concern was addressed in our study, which found that patients who received washed RBCs had significantly fewer effective blood transfusions than those who received non-washed RBCs. We then compared the physiological and biochemical parameters between the two groups and found no significant differences, which is consistent with previous studies. However, we believe that these findings do not accurately reflect the quality of RBCs. To investigate the factors affecting blood transfusion, we focused on the levels of CD47, PS, and vesicle secretion. We hypothesised that examining RBC immune function would reveal factors more accurately associated with RBC quality.

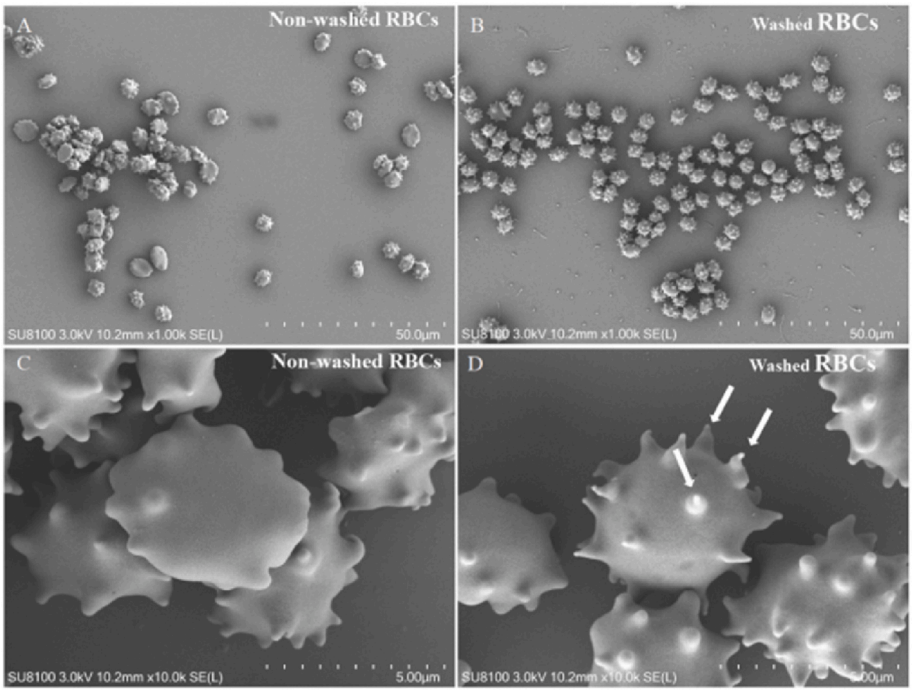

Fig. 4. Scanning electron micrographs of washed RBCs and non-washed RBCs. (**A,B**) The micrograph shows washed RBCs with more spherocytes and echinocytes on low magnification 1000×; (**C,D**) Washed RBCs show a higher degree of echinocytosis as indicated by arrows on high magnification 10,000×.

CD47 is a well-known integrin-associated transmembrane protein that plays a key role as a self-marker for RBCs [21]. It is a 50-kDa plasma membrane protein that has an extracellular immunoglobulin-like domain, five transmembrane domains, and a short cytoplasmic tail [22]. In phagocytic cells, CD47 serves as both a thrombospondin 1 (TSP1) receptor and a ligand for the inhibitory receptor signal regulatory protein (SIRP). Studies have confirmed that binding of the CD47 ligand with the SIRP receptor on macrophages and dendritic cells produces an inhibitory signal known as the "don't eat me" signal [23]. RBCs may be identified by their surface marker CD47, and in CD47-deficient mice splenic macrophages eliminate RBCs [24]. In other words, RBCs lacking CD47 are not recognised by macrophages and are phagocytosed as foreign bodies. Therefore, RBC survival in the human body is closely related with CD47 expression. Our results showed that none of the RBCs had a discernible change in CD47 expression. Therefore, we focused on CD47-positive RBCs that were non-apoptotic. We believe that the RBC status is closely related to the efficacy of transfusions. CD47 expression decreased significantly in non-apoptotic washed RBCs, and the rate of decline was more rapid as storage duration increased. This suggests that washing affects the expression of CD47 in RBCs and may reduce their quality.

The cell membrane is composed of a phospholipid bilayer, with phosphatidylserine (PS) normally kept on the inner side of the cell membrane [25]. When the cell membrane structure changes, PS is selectively oxidised and then integrated into the outer cell membrane [26]. The exposed PS is specifically recognised and bound by the macrophage surface receptor, resulting in the phagocytosis signal known as "eat me" [27]. During in vitro storage of RBCs, with ageing and steady energy consumption, the structure of the cell membrane is prone to molecular rearrangement, resulting in changes in morphology and function, as well as gradual exposure of PS molecules to the outer membrane [28]. If RBCs with high PS expression are transfused during clinical treatment, their scavenging ability is used to destroy a large number of RBCs following transfusion [29]. Our study found that washed RBCs exhibited higher PS expression, indicating that washing increased RBC apoptosis and had a significant impact on the RBC quality.

During normal RBC ageing, the budding of the plasma membrane, caused by complement-mediated calcium influx, produces microparticles and micro-vesicles, which are thereafter shed [30]. In vitro, RBC-derived extracellular vesicles (REVs) accumulate in stored blood products, which may induce proinflammatory and procoagulant effects and contribute to adverse transfusion events. Therefore, REV formation is closely related to RBC quality [31]. Our findings showed that washing increased the number of spherocytes and echinocytes, and at high magnification, RBCs appeared to be more prone to REV formation. These results do not indicate the maintenance of RBCs quality.

In this study, we systematically examined the effectiveness of blood transfusions in recipients of various types of RBCs and found that patients who received unwashed RBCs had better therapeutic outcomes than those who received washed RBCs. We also compared physiological and biochemical parameters between the two groups and found no significant differences. Thus, we believe that physiological and biochemical parameters are no longer sufficient to assess RBC quality. Changes in RBC immune function are closely linked to the efficacy of blood transfusions. Therefore, we focused on the changes in immune molecules in RBCs after washing. The findings revealed that washing reduced the expression of CD47 in nonapoptotic RBCs while increasing the expression of PS, providing a

new direction for studying the variables underlying the effects of washing RBCs during transfusion. Furthermore, washing intensified the changes in cell membrane morphology and increased the formation of micro-vesicles, making it difficult to complete transfusion therapy.

Undoubtedly, the effectiveness of blood transfusion is closely related to the course and status of patients, rather than just the quality of RBCs. In this regard, we intend to increase the number of samples in future trials and conduct a comprehensive assessment of additional parameters such as recurrence, complications, cure, infection, and mortality rates. In addition, owing to difficulties in material acquisition, the number of RBCs used to evaluate immune status after washing was insufficient. In future investigations, we intend to increase the sample size and examine additional relevant immunological indicators.

5. Conclusions

CD47 and PS are classical signalling molecules of RBCs that were significantly differentially expressed in the two blood-receiving groups. This may account for differences in the effectiveness of blood transfusions. Therefore, conventional parameters used in the past may not have been sufficient to fully evaluate the quality status of RBCs. With the progress and development of precision medicine, blood transfusion therapy cannot remain at the level of simply replenishing Hb and oxygen. The immune index of RBCs is expected to become a marker for evaluating quality status, to further predict the efficacy of blood transfusion and achieve better precision therapy. Collectively, our study provides a good reference for the development of new markers of the quality of RBCs.

Data availability statement

Data included in article/supp. Material/referenced in article, further inquiries can be directed to the corresponding author.

Funding statement

This research was funded by the Military Medical Service Project (NO. 20WQ028), Nanjing Health Science and Technology Development Project (YKK23224).

Ethics statement

This study was reviewed and approved by the Ethics Committee of the Jinling hospital, with the approval number: 2022DZGZR-012.

Informed consent statement

We've obtained consents from the voluntary blood donors. Written informed consent from patients was not obtained because this study is a secondary analysis of the data collected routinely for the purpose of evaluating the effectiveness of blood transfusions. Our research did not disclose any results related to the patient's personal identification information.

CRediT authorship contribution statement

Guangchao Zhao: Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Hongmei Zhang:** Validation, Supervision, Funding acquisition. **Xiaojun Kong:** Validation, Supervision, Funding acquisition. **Qing Qi:** Formal analysis, Data curation. **Tao Hou:** Formal analysis, Data curation. **Pingping Mao:** Formal analysis, Data curation. **Jianfeng Luan:** Resources, Methodology, Funding acquisition. **Wei Wang:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

None.

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