Int J Clin Exp Med 2015;8(10):18066-18073
www.ijcem.com /ISSN:1940-5901/IJCEM0013030

Original Article

Changes in platelet function following cold storage of RBC suspensions

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Received July 17, 2015; Accepted October 9, 2015; Epub October 15, 2015; Published October 30, 2015

Abstract: Objective: To provide a basis for the cold-storage of human platelets as a way to assess changes in platelet function. Methods: Red blood cell suspensions (11 U and 50 U) were randomly selected at different storage times (3-28 days) and evidence of platelet activation (CD62P) and thromboelastography (TEG) reaction times were investigated. Results: After 21 days of storage at 4°C, a large number of activated platelets (PAC1+62P+, PAC1-62P+) within the red blood cell suspension (RBCs) retained their function and had TEG-maximum amplitude (TEG-MA) indices in the normal range. Conclusion: We report that platelets in RBC suspensions retain high activity when stored at 4°C for 21 days. The results provide important information for studies that involve storing platelets under cold conditions.

Keywords: RBC suspensions, thromboelastography, blood platelets, cold storage, hemostasis

Introduction

In a clinical setting, a blood cell transfusion is mainly used to treat anemia; whereas platelets and fresh frozen plasma (FFP) are used in the treatment of thrombocytopenia and coagulopathy. However, some studies report that red blood cells suspensions (RBCs) promote hemostasis by platelet marginalization [1] and reduce the risk of bleeding in patients who received transfusions for anemia and thrombocytopenia [2-9]. This suggests that RBCs play a role in hemostasis.

In our early studies, we found that the hemostatic effect of RBC suspensions required platelets and residual coagulation factors. In order to determine whether platelets remain function in a cold RBC we assessed changes in blood routine, coagulation, and TEG indices at different storage times. Our work could potentially clear the misconception that platelets disintegrate and lose their function when stored under cold conditions.

Materials and methods

Preparation of red blood cell suspensions

Red blood cells suspensions (RBCs) were provided by the Shaanxi Provincial Blood Center and processed following standard protocols. All blood donors adhered to the Blood Donation Law of the Ministry of Health of the People's Republic of China. Venous blood samples were collected by vein puncture at the antecubital fossa into a 400-ml polypropylene bag containing 56 ml citrate phosphate dextrose (CPD). To separate plasma from RBCs, blood samples were centrifuged for 8 min at 4400 × g and the plasma was isolated using an automatic blood component separator (Sepamatic-SL (III) (Gemany, LMB). These procedures yielded approximately 200 ml plasma was collected. RBCs were suspended in 100 ml red blood cell

Table 1. Changes in blood routine, blood coagulation, and TEG indices from RBC suspensions with different storage times

	Length of storage at 4°C					*P*-value
	3 d	7 d	14 d	21 d	28 d	
RBC (× 10^{12}/L, $\bar{x}$±s)	6.81±0.65	6.98±0.74*	7.06±0.73*	7.02±0.77*	7.17±0.89*	0.000
Hb (g/L, $\bar{x}$±s)	209±18	211±17*	213±18*	211±18*	214±19*	0.000
Hct (L/L, $\bar{x}$±s)	0.634±0.04	0.658±.049*	0.677±0.054*	0.671±0.056*	0.697±0.075*	0.000
PLT (× 10^{9}/L, $\bar{x}$±s)	288±69	290±62	269±80	220±62*	194±46*	0.000
PT (s, $\bar{x}$±s)	16.6±4.4	16.4±2.3	19.2±2.7	22.4±3.4*	27.8±3.2*	0.001
APTT (s, $\bar{x}$±s)	66.7±25.2	69.3±25.4	73.0±27.6	82.9±15.9*	114.7±23.5*	0.000
INR ($\bar{x}$±s)	1.35±0.34	1.34±0.18	1.56±0.20	1.80±0.26*	2.22±0.24*	0.001
FIB (g/L, $\bar{x}$±s)	0.86±0.40	0.93±0.34	0.87±0.29	0.83±0.38	0.95±0.34	0.001
R (min, $\bar{x}$±s)	9.6±3.5	8.3±1.6	9.7±2.5	10.5±3.2	12.5±3.9*	0.000
K (min, $\bar{x}$±s)	9.6±7.6	5.4±2.5	6.7±3.3	9.9±5.3	12.4±7.2	0.000
Angle ($\bar{x}$±s)	47.8±7.78	53±8.3*	50.4±6.5*	46.9±6.7	44.9±6.3*	0.000
MA (mm, $\bar{x}$±s)	36.1±14.1	41.9±12.7*	40.6±11.6*	36.4±9.8	33±11.5	0.000
CI ($\bar{x}$±s)	-9.2±6.9	-5.5±3.1*	-7.4±4.1	-10.4±5.3	-13.1±6.7*	0.000

TEG = Thromboelastography; Hb = hemoglobin concentration; RBC = red blood cell count; PLT = platelet count; Hct = hematocrit; PT = prothrombin time; INR = international normalized ratio; a PTT = activated partial thromboplastin time; FIB = fibrinogen concentration; R = reaction time; K = kinetics,clot formation time, a (angle) = slope between r and k ; MA = maximum amplitude; CI = coagulation index. *Compared with 11U RBC suspension on Day 3, $P < 0.05$. The *P*-value denotes repeated measurements.

additive solution containing MAP and stored 4°C for 35 days. In order to ensure the effectiveness of the blood components, each blood component was stored under the appropriate conditions. The suspended red blood cells were cold stored at 4°C, plasma was frozen stored at -20°C, and the platelet suspensions were concussion stored at 22°C [10].

Sampling of the suspended red blood cells

RBC suspensions were divided into two groups (11 U and 50 U) for either continuous observation or random selection. Blood samples under continuous observation were assessed for changes in routine, coagulation, and TEG indices following storage at 4°C for 3, 7, 14, 21, and 28 days. Other blood samples were randomly selected from the department blood refrigerator (storage time: 3-33 days) and heated. Blood cell morphology was observed using an Olympus-BX53 microscope and platelet activation was confirmed by flow cytometry. The Shaanxi Provincial People's Hospital ethics committee and the Shaanxi Provincial Blood Center approved all experiments.

Preparation of platelet-rich plasma (PRP)

Platelet-rich plasma (10 ml) from the 11 U RBC suspension (continuous observation) was centrifuged at 1000 × g for 12 min and absorbed. The supernatant and tunica albuginea parts were combined to evaluate TEG and blood routine indices.

Blood analyses

Routine blood analyses measured the RBC count, hematocrit (Hct), hemoglobin content (Hb), and platelet (PLT) count using an automatic hemacytomer (Coulter LH 750). The Sysmex CA-7000 coagulation analyzer measured prothrombin time (PT), activated partial thromboplastin time (APTT), international standardization ratio (INR), thrombin time (TT), and plasma fibrinogen levels (FIB). The thromboelastography (TEG) parameters recorded as reaction times (R or R-time), K times (K or K-time), Angle, maximum amplitude (MA), and coagulation index (CI). These measurements were gathered using the TEG 5000 Thromboelastograph Hemostasis Analyzer (Haemoscope Corp., IL). Expression of platelet markers CD61, PAC-1,

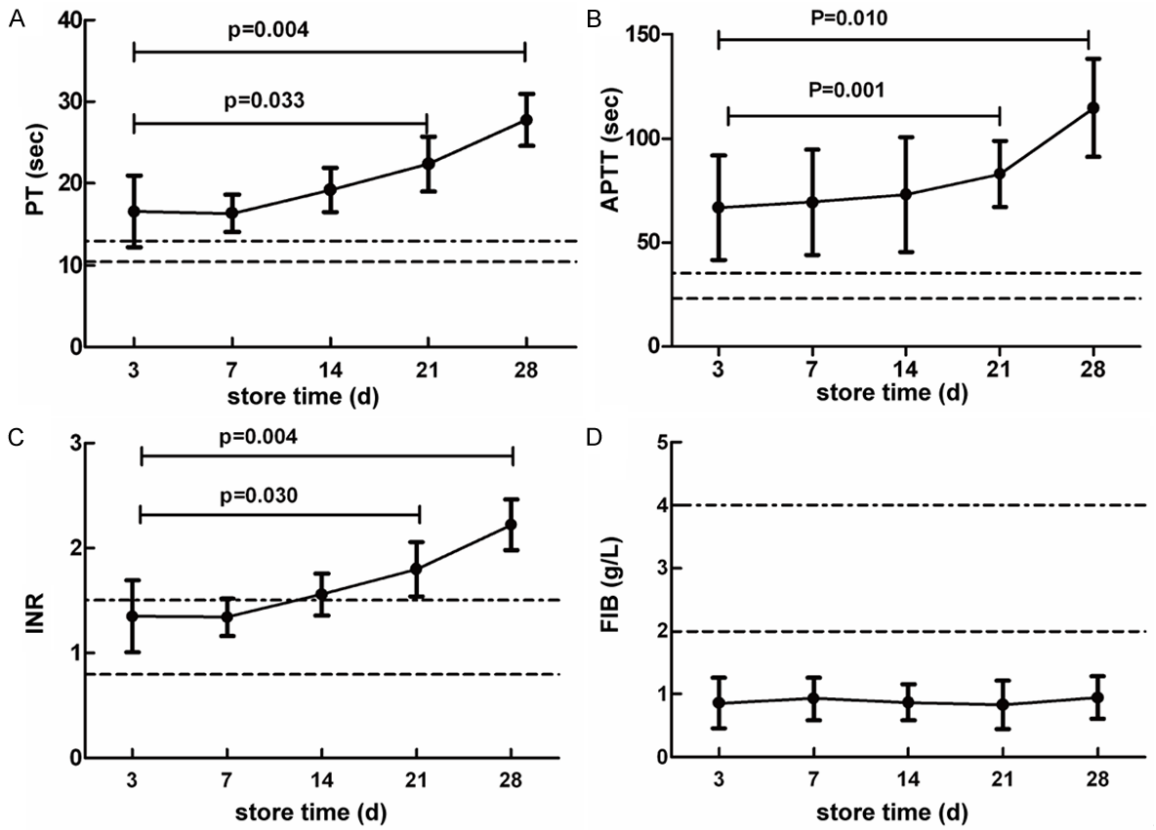

Figure 1. Changes in blood coagulation indices of (11 U) RBC suspensions. Graphs shows all prothrombin times (PT) are above the normal range for RBCs with a significant change in PT on Day 21 ($P < 0.05$) (A). Activated partial prothromblastin times (APTT) increase significantly on Day 21 (B). The international standardization ratio (INR) remains within the normal range until Day 14. Then a significant increase occurs on Day 21 (C). Plasma fibrinogen levels stayed below normal during the 4°C storage period (D). Dotted line represents the normal range.

and CD62P were detected by flow cytometry (Becman Coulter FC 500) and CXP software.

Statistical analysis

Data were analyzed using the SPSS statistical software (version 18.0). Analysis of parametric data was performed by the paired Student's t-test. An association between categorical variables was tested by the Chi-square test and a repeatable ANOVA. Statistical significance was set at $P < 0.05$.

Results

Routine blood indices remained at or above the normal range after cold storage

As shown in **Table 1**; **Figures 1** and **2**, when RBC suspensions were stored at 4°C the number of RBCs increased, while the number of platelets gradually decreased over time. Additionally, blood coagulation PT and APTT surpassed the normal range at Day 3 and continued until Day 21. After Day 14, the INR values extended beyond the normal range and FIB levels remained below the normal range. The TEG-reaction times (TEG-R) reached beyond the normal range at Day 21. The TEG K-times (which reflect FIB levels) were beyond the normal range. TEG-Angle (reflects blood coagulation rates), MA values (reflects platelet function), and CI (reflects overall coagulability), were beyond the normal range with low coagulation states.

TEG indices of platelet-rich plasma (PRP) remain in the normal range for 21 days

As shown in **Table 2**, PRP obtained from RBC suspensions stored at 4°C for 14 days had a

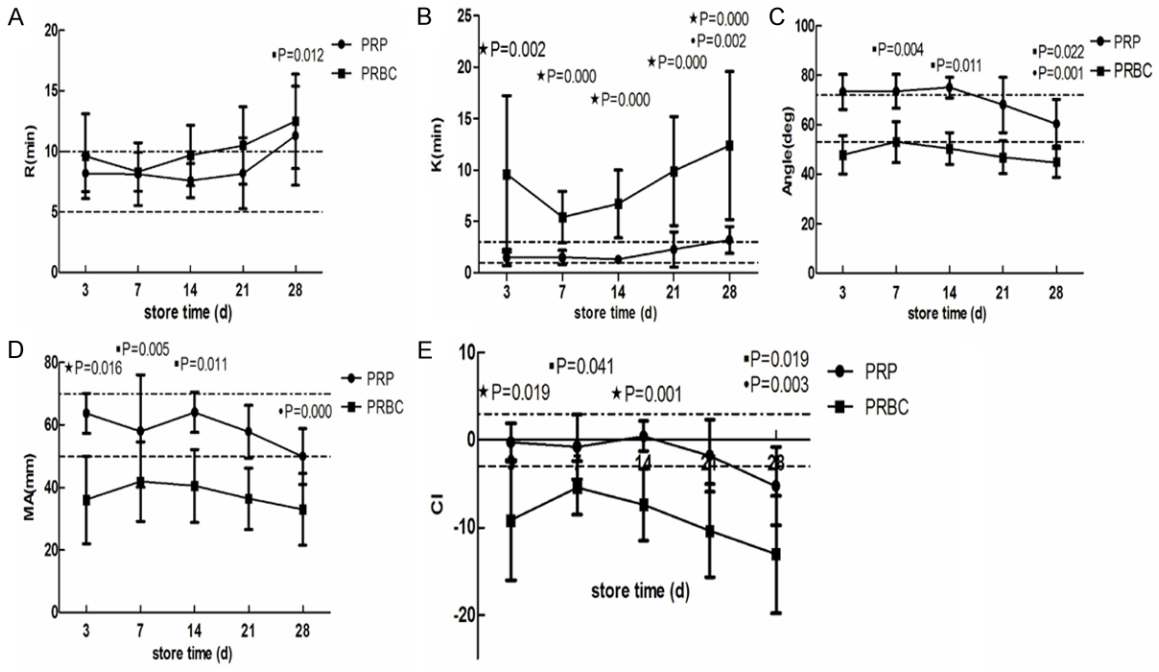

Figure 2. Comparison of TEG indices of RBC suspensions and platelet-rich plasma. TEG R-times (A), TEG K-times reflect plasma fibrinogen levels (B), TEG-Angle reflect coagulation rates (C), TEG-MA values reflect platelet function (D), and TEG-CI reflects overall blood coagulation rates (E) for RBCs and platelet-rich plasma. Dotted line represents the normal range. *RBCs versus PRP; ● versus 3 days of the PRP storage; ■ versus 3 days of RBCs storage.

Table 2. TEG indices of platelet-rich plasma with different storage times

	Length of storage at 4 °C					*P*-value
	3 d	7 d	14 d	21 d	28 d	
RBC (× 10^{12}/L, $\bar{x}$±s)	0.25±0.11	0.18±0.07	0.24±0.06	0.26±0.15	0.47±0.23*	0.000
Hb (g/L, $\bar{x}$±s)	5±4	3±2	4±2	7±3	10±3*	0.000
Hct (L/L, $\bar{x}$±s)	0.022±0.012	0.015±0.007	0.021±0.005	0.027±0.011	0.081±0.149	0.004
PLT (× 10^{9}/L, $\bar{x}$±s)	525±174	581±146	427±186	237±123*	399±152	0.000
R (min, $\bar{x}$±s)	8.2±1.5	8.1±2.6	7.6±1.4	8.2±2.9	11.3±4.1	0.000
K (min, $\bar{x}$±s)	1.5±0.8	1.5±0.7	1.3±0.4	2.3±1.7	3.2±1.3*	0.000
Angle ($\bar{x}$±s)	73.3±7.2	73.5±6.8	75.1±4.2	68.1±11.2	60.3±9.9*	0.000
MA (mm, $\bar{x}$±s)	63.8±6.4	58.1±18	64.1±6.5	57.9±8.5	50±8.9*	0.000
CI ($\bar{x}$±s)	-0.3±2.2	-0.8±3.7	0.4±1.7	-1.8±4.1	-5.3±4.5*	0.037

TEG = Thromboelastography; Hb = hemoglobin concentration; RBC = red blood cell count; PLT = platelet count; Hct = hematocrit; R = reaction time; K = kinetics, clot formation time, a (angle) = slope between r and k; MA = maximum amplitude; CI = coagulation index. *Compared with Day 3, $P < 0.05$. The *P*-value denoted repeated measurements.

gradual decrease in the number of platelets. TEG R-times and TEG coagulation indices (TEG-CI) were within the normal range for 21 days, and then dropped below normal on Day 28. However, TEG K-times TEG-Angle, and TEG-MA levels remained within the normal range for the entire storage period.

Comparison of TEG indices for RBC suspensions and platelet-rich plasma (PRP)

As shown in **Figure 2**, there were no statistical differences between RBCs R-times and PRP R-times Day 28. However, there was a significant difference between RBC K-times and PRP

Table 3. Expression level of CD62P RBC suspensions with different storage times

	< 7 d	8-14 d	15-21 d	≥ 22 d
$PAC1^+62P^-$ (%, $\bar{x}\pm s$)	6.84±5.61	3.81±3.93	1.81±3.92	2.36±7.55*
$PAC1^+62P^+$ (%, $\bar{x}\pm s$)	34.02±18.45	41.74±22.25	33.92±12.91	22.57±8.2
$PAC1^-62P^+$ (%, $\bar{x}\pm s$)	42.11±17.11	43.26±25.84	50.37±17.94	60.41±18.65*
R (min, $\bar{x}\pm s$)	8.8±2.2	9±1.2	9.6±1.8	14±5.3*
K (min, $\bar{x}\pm s$)	4.9±3.4	4.7±2.7	6.1±3.8	10.5±6.8*
Angle ($\bar{x}\pm s$)	56.9±10	55.5±6.5	53.5±6.8	47.2±7.3*
MA (mm, $\bar{x}\pm s$)	47.3±13.5	49.4±8.9	43.4±10.9	35.7±10.5*
CI ($\bar{x}\pm s$)	-5.3±5	-5.2±3.1	-7±3.9	-12.5±6.4*
PLT ($\times 10^9$/L, $\bar{x}\pm s$)	213±75	199±69	156±41	192±73
Hb (g/L, $\bar{x}\pm s$)	129±57	139±50	145±52	168±49
RBC ($\times 10^{12}$/L, $\bar{x}\pm s$)	4.47±2.28	4.49±1.67	4.62±1.65	5.49±1.64
Hct (L/L, $\bar{x}\pm s$)	0.413±0.198	0.435±0.161	0.449±0.166	0.545±0.164

PAC1 = an activated complex on the glycoprotein II b/III a; CD62P = P-selectin; Hb = hemoglobin concentration; RBC = red blood cell count; PLT = platelet count; Hct = hematocrit; R = reaction time; K = kinetics, clot formation time, a (angle) = slope between r and k; MA = maximum amplitude; CI = coagulation index. *Compared with Day 7, $P < 0.05$. The normal range for $PAC1^+62P$ goes as follows: $PAC1^+62P^- < 10\%$; $PAC1^+62P^+ < 10\%$; $PAC1^-62P^+ < 4\%$.

K-times. The RBCs K-time was above the normal range while the PRP K-times were within the normal range. The TEG-Angle values, which reflected the blood coagulation rate, as well as the PRP-Angle and RBCs-Angle values were in the vicinity of the normal range on both sides and there was no statistical difference. As for the MA values (PRP-MA), which reflect platelet function, and the CI value (PRP-CI), which reflects whole blood coagulation rates, they were in the normal range. As for RBCss, the coagulation indices (CI) and maximum amplitude levels (MA) were below the normal range in a low coagulation state.

Platelets remain unaffected by a 3-week storage period under cold conditions

The expression levels of activated platelet marker, $PAC1^-62P^+$, in suspended red fell below 50% when stored for less than 14 days at 4°C (**Table 3** and **Figure 3**). By Day 22 CD62P expression levels increased. This data suggests that platelets remained active.

Blood smear of RBC suspensions reveal sustained presence of platelets

Wright-Geima staining revealed that a large number of platelets remained in the RBC suspension at Day 21 and decreased by Day 28 (**Figure 4**).

Discussion

The goal of this study was to validate the presence of high platelet activity in suspended red blood cells stored under cold storage conditions. Our data would provide some important information for studies on the cold storage of platelets. Previous reports show that researchers believed that cold storage of red blood cells could cause the platelets to be rapidly eliminated *in vivo*, so they suggested the platelets should be preserved in 20-24°C oscillating conditions [10, 11-20]. Further improvement of platelet additive solutions and bags has extended the platelets storage time to 5-7 days [21-26]. In our study, the platelets in suspended red blood cells retained their aggregate function in a highly activated state in cold storage for 21 days. Additionally the platelets retained certain functions after 28 days of cold storage. These results provide the basis for 4°C cold-storage platelets.

A large number of platelets contained in suspended red blood cells, the amount of platelets ($288\pm69 \times 10^9$ cells/l) remained in the RBC suspension after Day 3 and gradually decreased ($194\pm46 \times 10^9$ cells/l) after Day 28. Furthermore, morphological examinations revealed that platelets maintained their cellular integrity until Day 21. In order to analyze the changes in platelet function, we prepared platelet-rich plasma (PRP) from suspended RBCs stored at 4°C for varying lengths of time. TEG index showed that the R-times, K-times, and Angle values, which reflect blood coagulation, were in the normal range during 21 days of storage. MA and CI values, which reflect platelet function and overall blood coagulation rate, respectively, were also in the normal range after 21 days of storage.

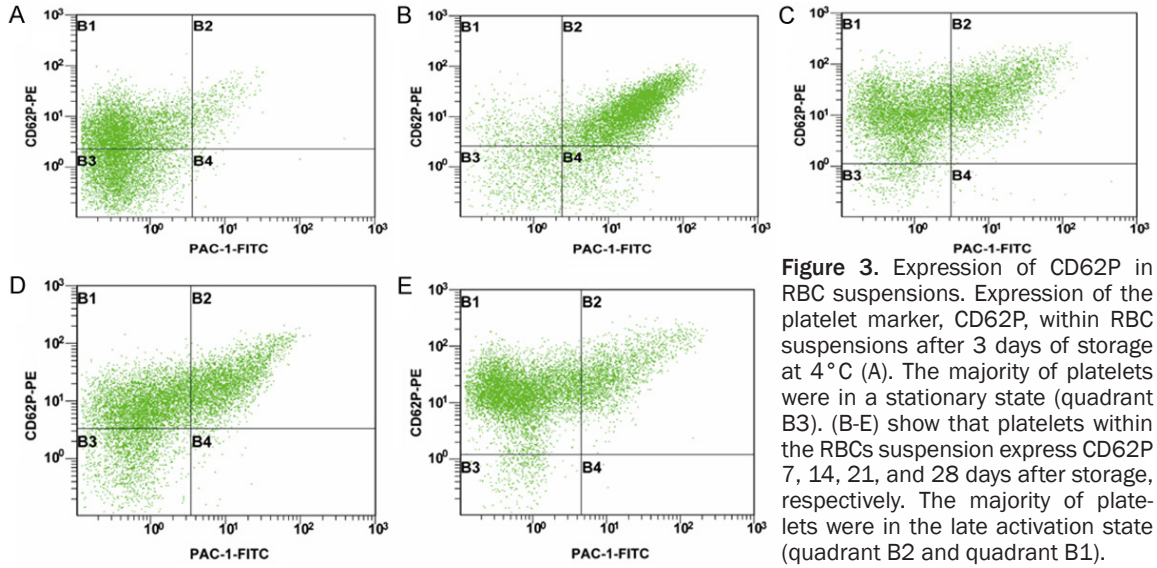

Figure 3. Expression of CD62P in RBC suspensions. Expression of the platelet marker, CD62P, within RBC suspensions after 3 days of storage at 4°C (A). The majority of platelets were in a stationary state (quadrant B3). (B-E) show that platelets within the RBCs suspension express CD62P 7, 14, 21, and 28 days after storage, respectively. The majority of platelets were in the late activation state (quadrant B2 and quadrant B1).

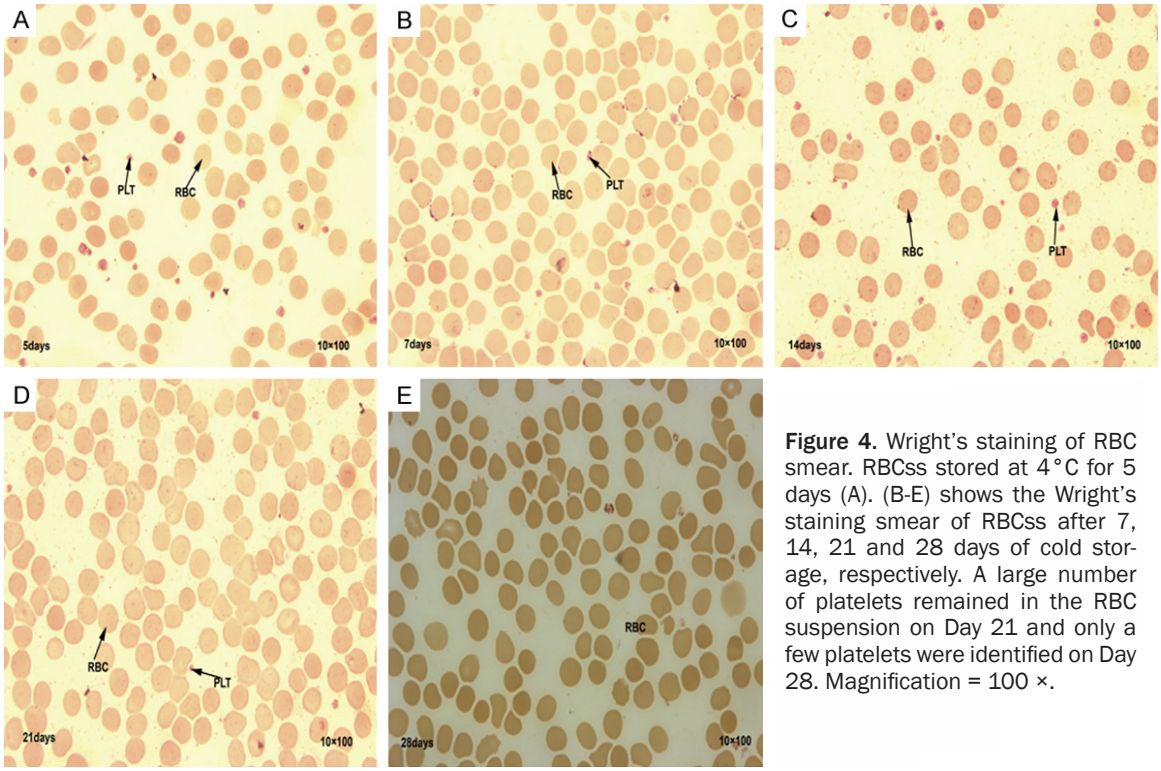

Figure 4. Wright's staining of RBC smear. RBCss stored at 4°C for 5 days (A). (B-E) shows the Wright's staining smear of RBCss after 7, 14, 21 and 28 days of cold storage, respectively. A large number of platelets remained in the RBC suspension on Day 21 and only a few platelets were identified on Day 28. Magnification = 100 ×.

Our results are consistent with previous findings from Stiegler *et al*., and Josefsson *et al*., in which the research groups believed that platelets retained certain activities under cold storage conditions, and suggests that storing platelets at 4°C is more beneficial than storage at 22°C, particularly for patients with acute bleeding [27, 28]. The underlying mechanism by which platelets retain their functional abilities is unclear, but Frederick *et al*., speculated that a specific molecule within the RBC supernatant inhibits platelet aggregation [29]. Additionally, the blood coagulation indices (PT, APTT, INR, and FIB) were in a low coagulation state. This is due to the dilution of coagulation factors and decreased activity during the preparation process. In this process many blood coagulation factors were removed as a considerable

amount of RBC maintenance solution was added. The TEG index in a low coagulation state, and the R, K, Angle, MA, and CI values were out of the normal reference range. This may be related to the decreased concentration of coagulation factors or may result from changes in red and white blood cells after cold storage.

Acknowledgements

This study was supported by a grant from the Shaanxi Provincial Blood Center to Shi-Hui Ye, Jing-Wen Zhao and Xiao-Li Cao.

Disclosure of conflict of interest

None.

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