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Original Article

Clinical practice guideline of transfusion: survival and oxygen-carrying capacity of red blood cells

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Abstract: This study aimed at investigating the effect of *in vitro* preservation on survival and oxygen-carrying capacity of RBCs and obtaining clear and definite function change of RBCs during their preservation. The transfusion of fresh red blood cells (RBCs) is recommended for transfusion-dependent patients. 0-day- and 35-day-stored RBCs are still equally used in clinical practice now, which may influence the therapy effect of blood transfusion. The survival of 3-day-, 10-day- and 21-day-stored RBCs after blood transfusion was determined by flow cytometry based on natural differences in RBC antigens between donors and patients. The effect of *in vitro* preservation on RBC oxygen-carrying capacity was assessed by determining p50 and effective oxygen-carrying amount with blood-gas analyzer. In this study, we found that the PTR of RBCs decreased with storage time increasing, and the mean 24-h PTR of 3-day-, 10-day-, and 21-day-stored RBCs complied with the standard of RBC viability (i.e. the survival of the cells at least 75 percent at 24 h after transfusion). The mean potential life span (MPL) and time to reach a PTR of 50 percent of the 24-h PTR (T50) of 3-day-, 10-day-, and 21-day-stored RBCs are almost identical. *In vitro* studies showed that both p50 and effective oxygen-carrying amount of RBCs decreased with storage time increasing. In addition, the number of survival cells in the different RBC suspensions with same total effective oxygen-carrying amount had no significant difference. These findings provide theoretical basis and practical direction for scientific and efficient blood transfusion.

Keywords: Red blood cell, transfusion, posttransfusion recovery, effective oxygen-carrying amount, p50

Introduction

During storage period, red blood cells (RBCs) undergo various structural and biochemical changes, which impair their oxygen-carrying ability and trigger secondary reactions [1, 2]. Increasing evidences show that a shorter RBC storage period is beneficial, and the use of fresh RBCs has been recommend for critically ill patients, patients undergoing surgery and transfusion-dependent patients [3-7]. However, the actual relationship between the storage lesions and RBC survival and function after transfusion remains unclear. At present, 0-day- and 35-day-stored RBCs are still equally used in clinical practice, which may influence the therapy effect of blood transfusion. Therefore, clear and definite function change of RBCs during their preservation has important guiding significance for clinical blood transfusion. Survival *in vivo* and oxygen-carrying capacity are very important parameters for evaluating function of RBCs.

The first standards for RBC storage are that the cells do not haemolyse in the bottle and they can circulate when they are reinjected into the donor or transfused into a recipient [8]. In a sense, these are still the only standards. Now, they are formalized in the US licensure requirements that survival of at least 75% exists 24 h after infusion and haemolysis must be less than 1% at the end of the approved storage period. Estimation of RBC survival *in vivo* is conventionally carried out by using cells labeled with radioisotopes, such as technetium-99 and chromium-51 [9]. Cells are labeled *in vitro*, injected into the subject, and then sequential blood samples are taken for gamma counting. Although this method has the advantage of being very sensitive, the subjects have to be exposed to some ionizing radiation. Thus this method is not allowed in many countries. As an alternative, flow cytometry method might be desirable. During the past two decades, flow cytometric determination of chimerism in RBC populations has been widely applied for the

Table 1. Patient characteristics

	Group 1	Group 2	Group 3
Sex (male/female)	10/10	8/12	9/11
Age (year)	37 ± 10	35 ± 14	39 ± 15
Hb (g/L)	55.2 ± 2.4	57.7 ± 2.5	55.4 ± 1.2
RBC (10^{12}/L)	1.69 ± 0.18	1.75 ± 0.08	1.54 ± 0.12
Rh phenotype	cCDEE	cCDEE	cCDEE
Pelvic fracture (n)	6	6	6
Intertrochanteric fracture (n)	2	2	2
Multiple fracture (n)	12	12	12

follow-up of patients after allogeneic marrow transplantation, used to determine and quantify fetal RBCs in fetomaternal hemorrhage, identify illicit homologous blood transfusion in athletes and monitor the survival of donor RBCs after transfusion [10-14]. Luten *et al.* reported survival of RBCs with different storage periods after transfusion [15]. There are various methods to evaluate oxygen-carrying capacity of RBCs. Among them, the most common method is to determine p50, which is the oxygen tension when hemoglobin (Hb) binding sites are 50% saturated and can reflect the oxygen affinity of RBCs [16]. The normal p50 in adults at sea level is 26.3 mmHg. In addition, effective oxygen-carrying volume is frequently detected to assess oxygen-delivering capacity of preserved RBCs.

In this study, we investigated the survival of 3-day-, 10-day- and 21-day-stored RBCs after blood transfusion by flow cytometry based on natural differences in RBC antigens between donors and patients. We also explored the effect of *in vitro* preservation on oxygen-carrying capacity of RBCs by determining p50 and effective oxygen-carrying amount. In addition, we further investigated the relationship between survival and effective oxygen-carrying amount of RBCs, and aimed to achieve function measurement of RBC infusion. These findings will provide theoretical basis and practical direction for scientific and efficient blood transfusion.

Materials and methods

Subjects

Between April 2014 and January 2015, 60 eligible orthopedic patients were included in this study. These patients had never been transfused previously or not received a transfusion less than 6 months before this study, and their Rh phenotype was cCDEE. In addition, except fracture, they had no other diseases. They were randomly divided into three groups, each with 20 cases. They all had serious anemia and were transfused with homotypic ABO blood. The patients' characteristics were listed in **Table 1**. The amount of RBC transfusion of every patient was 8×10^8 cells per ml of patient's blood volume (BV). Patient's BV was calculated according to the following equation (Eq. 1).

Male: $BV\ (ml) = \exp [7.0506 + 0.724 \times (0.00718 \times height\ (cm)^{0.725} \times weight\ (Kg)^{0.425}]$

Female: $BV\ (ml) = \exp [6.9870 + 0.724 \times (0.00718 \times height\ (cm)^{0.725} \times weight\ (Kg)^{0.425})]$

The three groups were transfused with 3-day-, 10-day-, and 21-day-stored RBCs for once, respectively. The Rh phenotype of blood donor was cCDee. After 126 days, those patients were redivided randomly into three groups for the second transfusion. The study was approved by the Ethics Committee of Henan Provincial People's Hospital. Written informed consent was obtained from each patient.

The preparation of red cell concentrates (RCCs)

RBCs were prepared as described previously with standard procedures [17]. Briefly, whole blood was collected in a quadruple citrate phosphate dextrose (CPD)-saline adenine glucose mannitol (SAGM) top-and-bottom bag system (Composelect, Fresenius Kabi, Bad Homburg, Germany). After cooling for 6 h and centrifugation, the blood was separated into plasma and RBCs by an automated blood processor (Compomat G4, Fresenius Kabi). SAGM was transferred from the RBC storage bag to the RBCs. Leukodepleted RCCs were obtained by using the CompoFlow Select in-line filtration system (Fresenius Kabi) and then were stored at 2 to 6°C for a maximum of 35 days. The amount of RBCs isolated from 200 ml whole blood was defined as one unit (U).

Flow cytometric determination of survival

Venous blood (3 ml) was withdrawn from every patient at 1 and 24 h and 7, 14, 21, 28, 56,

84 and 126 days after transfusion and added into PE tubes containing CPD. Before labeling, white cells were removed using Ficoll-Paque centrifugation (Amersham Biosciences, Uppsala, Sweden). Briefly, the blood was mixed with same amount of phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). This mixture was layered over Ficoll-Paque and centrifuged (400×*g*, 30 min, 25°C). The RBC pellet was washed three times in PBS containing 0.1% BSA. A RBC suspension with 1.25×10^8 cells/mL was prepared. RBCs were labeled with human anti-E antibody directed against the E antigen by adding 50 μL specific antiserum to 200 μL RBC suspension. After incubation for 1 h at 37°C, the RBCs were washed three times in PBS containing 0.1% BSA to remove unbound antibodies. Then 7.5×10^5 RBCs were pelleted in round-bottomed 96-well microtiter plates and resuspended in 70 μL of a 1:128 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-human IgG-Fab (Cappel, Durham, NC, USA). RBCs were incubated for 30 min at 25°C in darkness, and then were washed three times in PBS containing 0.1% BSA. After being washed, the RBCs were resuspended in 1 mL PBS containing 0.1% BSA. The number of antigen-positive RBCs was determined by flow cytometer (Cytomics FC 500; Beckman Coulter, Inc., Fullerton, CA, USA). The posttransfusion recovery (PTR) was calculated according to the following equation (Eq. 2).

$$PTR\ (\%) = N \times BV/RBC\ number \quad (2)$$

where N was the number of antigen-positive RBCs per ml of blood, BV was the patient's blood volume and RBC number was the number of RBCs transfused into the patient.

In addition, two other characteristics of RBC survival, mean potential life span (MPL) and time to reach a PTR of 50 percent of the 24-h PTR (T50), were calculated from the survival data. T50 and MPL were calculated according to the equation of the regression line on the basis of blood samples collected 24 h after transfusion until 126 days after transfusion, which all data were recalculated relative to a 24-h PTR set at 100 percent. The equation of the regression line follows the quadratic equation of $y = ax^2 + bx + c$, where a, b, and c were determined for every patient.

Determination of p50 and effective oxygen-carrying amount

Effective oxygen-carrying amount is that 100 mL whole blood indeed sends the oxygen amount to tissue in a blood circulation under normal physiological condition and standard pressure. The oxygen-carrying amount of 1 g hemoglobin (Hb) is 1.34-1.36 ml O_2. Per 100 ml whole blood contains about 15 g Hb, which carries about 20 ml O_2. To determine effective oxygen-carrying amount *in vitro*, the packed RBCs with different storage periods (0, 3, 7, 10, 14, 21, 28 and 35 days) were resuspended in plasma to obtain 3 ml RBC suspensions with 3.5×10^{12} cells/mL. Antifoaming agent (20 μL) was added to each RBC suspension. Then gas mixture consisting of 11.5 percent O_2, 2.2 percent CO_2 and 86.3 percent N_2 was supplied to the RBC suspension at a flow rate of 100 ml/min, and the change of oxygen partial pressure was determined by blood-gas analyzer (Model ABL-3, Radiometer Inc., Westlake, Ohio, USA). When oxygen partial pressure reached 100 mmHg (arterial oxygen tension), oxygen saturation was determined and recorded as S1. Accordingly, the gas mixture consisting of 3.5 percent O_2, 1.8 percent CO_2 and 94.7 percent N_2 was supplied to the RBC suspension at a flow rate of 100 ml/min. Oxygen saturation was determined at an oxygen partial pressure of 40 mmHg (partial venous oxygen pressure), and recorded as S2. Effective oxygen-carrying amount was calculated according the formula: Effective oxygen-carrying amount = 20 × (S1-S2). p50 is the partial pressure of oxygen, corresponding to 50% of Hb saturation with oxygen, and can be obtained from the blood gas analysis at an oxygen partial pressure of 100 mmHg.

Statistical analysis

Statistical analyses were carried out on the GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). All results are expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) or student's *t*-test was used to analyze significance. Statistical significance was presumed when $P<0.05$.

Results

Survival and PTR of RBCs after transfusion

The three groups of patients were transfused with 3-day, 10-day, and 21-day-stored RBCs,

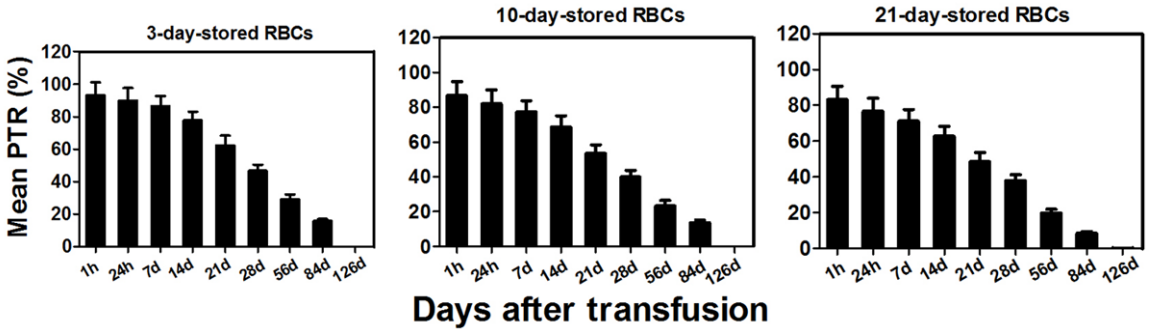

Figure 1. The mean PTR of 3-day-, 10-day- and 21-day-stored RBCs at 1 and 24 h and 7, 14, 21, 28, 56, 84 and 126 days after transfusion.

Table 2. RBC survival data

Survival[a,b]	3-day stored RBCs	10-day stored RBCs	21-day stored RBCs
24-h PTR (%)	89.8 ± 7.8[c,d]	81.9 ± 8.1[c]	76.7 ± 7.2
T50 (days)	41 ± 14	41 ± 13	41 ± 10
MPL (days)	118 ± 15	114 ± 16	114 ± 18

[a]n = 20 for 24-h PTR; n = 19 for T50 and MPL. Data are mean ± SD. [b]T50 and MPL are the values of the RBCs surviving the first 24 hours after transfusion. [c]Significantly different from 21-day stored RBCs, $P<0.05$. [d]Significantly different from 10-day stored RBCs, $P<0.05$.

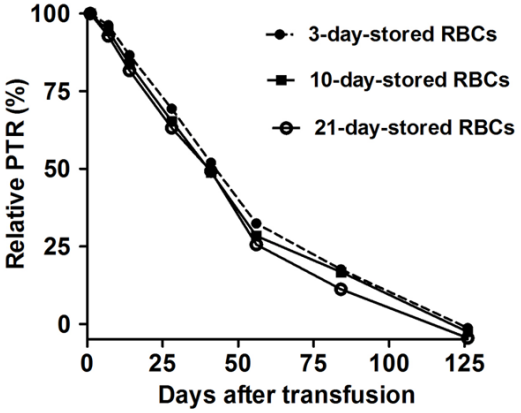

Figure 2. The relative mean PTR of 3-day-, 10-day- and 21-day-stored RBCs at several time points after transfusion (24 h and 7, 14, 21, 28, 56, 84 and 126 days). The relative PTR is the recovery of RBCs after setting the 24-h PTR at 100 percent.

respectively. Then 3 ml venous blood was withdrawn from every patient at 1 and 24 h and 7, 14, 21, 28, 56, 84 and 126 days after transfusion, and the number of antigen-positive RBCs was determined by flow cytometry. Then the PTR was calculated according to the Eq. 2. The results showed that the PTR of RBCs decreased with storage time increasing. The mean 1-h PTR of 3-day-, 10-day-, and 21-day-stored RBC was 93.2%, 86.8%, and 83.2%, respectively, and the mean 24-h PTR was 89.8%, 81.9% and 76.7%, respectively (**Figure 1**). The mean 24-h PTR of RBCs of the three storage periods was statistically within the required limit of 75 percent.

T50 and MPL of RBCs after transfusion

T50 and MPL are two important parameters of RBC survival, and calculated from the equations for the regression lines based on the 24-h recovery, that is, after setting the 24-h PTR at 100 percent. The T50 of 3-day-, 10-day-, and 21-day-stored RBCs was 41 days. The MPL of 3-day-, 10-day-, and 21-day-stored RBCs was 118, 114 and 114 days, respectively. The T50 and MPL of RBCs of the three storage periods are almost identical (**Table 2** and **Figure 2**).

p50 and effective oxygen-carrying amount

We determined the p50 and effective oxygen-carrying amount of RBCs with different storage periods *in vitro* by blood gas analyzer. As shown in **Figure 3A**, p50 decreased with storage time increasing, which indicated that oxygen affinity of Hb decreased with storage time increasing. Furthermore, the correlation between p50 and storage time was obtained by regression analysis. The equation of the regression line was the linear equation of $y = -0.2588x + 28.35$, where y was the p50 (mmHg), and x was the storage time (day), and the correlation coefficient was -0.990 ($P<0.0001$). Accordingly, the effective oxygen-carrying amount of RBCs decreased with the preservation lasting, and the correlation between the effective oxy-

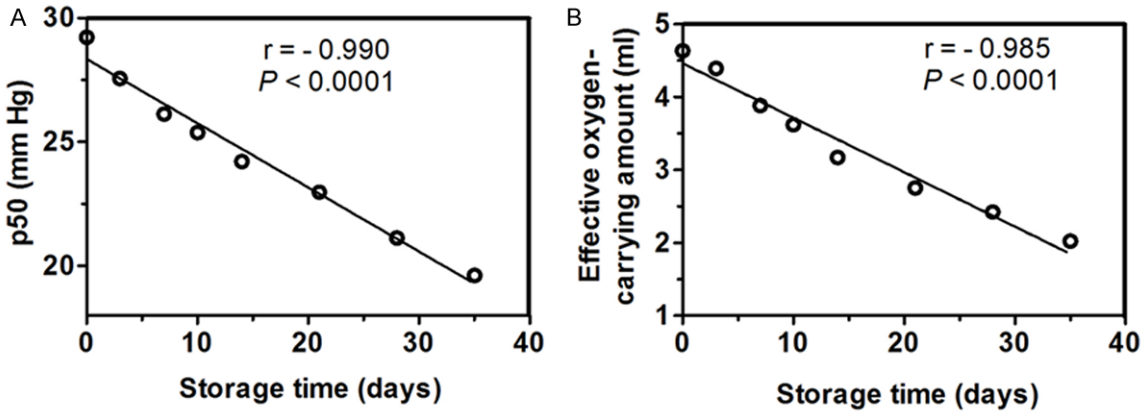

Figure 3. The p50 and effective oxygen-carrying amount of RBCs with the different storage periods. A. p50 of stored RBCs decreased with storage time increasing. B. The effective oxygen-carrying amount of stored RBCs decreased with the preservation lasting.

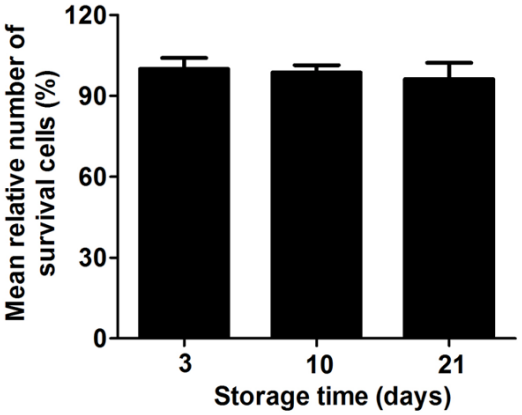

Figure 4. The number of survival cells of 3-day-, 10-day- and 21-day-stored RBCs which had same total effective oxygen-carrying amount had no significant difference.

gen-carrying amount and storage time was obtained by regression analysis (**Figure 3B**). The equation of the regression line was the linear equation of y = -0.0746x + 4.461, where y was the effective oxygen-carrying amount (ml), and x was the storage time (day), and the correlation coefficient was -0.985 (P<0.0001).

Correlation between survival and effective oxygen-carrying amount

Our *in vivo* studies showed that the mean 24-h PTR of RBCs decreased with storage time increasing, and 24-h PTR of 3-day-, 10-day-, and 21-day-stored RBCs was 89.8%, 81.9% and 76.7%, respectively. Then we also performed the *in vitro* studies and found the effective oxygen-carrying amount of RBCs decreased with storage time increasing. We further investigated the correlation of survival and the effective oxygen-carrying amount. According to the regression equation about the effective oxygen-carrying amount and the storage time, we set the effective oxygen-carrying amount of the 1 U 3-day-stored RBCs as total effective oxygen-carrying amount, and calculated the number of 10-day- and 21-day-stored RBCs, respectively. Then the RBC suspensions of the three different storage periods which had same total effective oxygen-carrying amount were transfused into the three treatment groups, respectively. The number of antigen-positive RBCs was determined by flow cytometry at 24 h after transfusion, and the total number of antigen-positive RBCs in each patient was calculated following the formula: Total number = N × BV, where N was the number of antigen-positive RBCs per ml of blood, and BV was the patient's blood volume. As shown in **Figure 4**, the mean total number of antigen-positive RBCs in the three treatment groups was almost identical, and had no significant difference. This result indicated that the different RBC suspensions with same total effective oxygen-carrying amount had almost identical number of survival cells that can circulate in the body after transfusion.

Discussion

Whole blood is collected into plastic packs with pre-measured anticoagulant-preservative

for cold preservation. The Hb content, preservative, volume, and storage interval or "shelf life" differ according to national standard [18]. During the storage period, the changes of other cells and plasma proteins are not synchronized with the change of RBCs, for example, granulocytes and platelets lose biological function within 48 h, and RBCs stored for a maximum of 35 days remain biological function [19]. Therefore, in practice, whole blood is used infrequently for situations such as massive hemorrhage where RBCs, plasma factors and volume are all needed.

RCCs are prepared by removing plasma from whole blood, replacing plasma with an additive solution to improve cell viability during extended storage period. RBCs age more quickly during refrigerated storage than they do in the body [20]. In storage period, RBCs change shape, become acidotic, lose adenosine triphosphate (ATP), 2,3-diphosphoglycerate acid (2,3-DPG) and membrane. Some break down, and some fail to circulate in the body [21]. These time-dependent changes in RBC quantity and quality are generally called the storage lesion. Owing to storage lesion, the RBCs with exceeding the shelf life have low survival and effective oxygen-carrying amount, and are not transfused. The gold standard of RBC viability is that the survival of the cells at least is 75 percent at 24 h after transfusion, which only permits a quarter of transfused RBCs to be non-viable. In addition, the notion that RBCs lose efficacy has been proposed based on claims that they do not circulate or they do not transport oxygen [22]. Therefore, survival and oxygen-carrying capacity are two important parameters for stored RBCs. In this study, we detected the survival of donor RBCs in patients by flow cytometry and calculated the PTR of stored RBCs. We observed that the mean 1-h PTR of 3-day-, 10-day- and 21-day-stored RBCs was 93.2%, 86.8%, and 83.2%, respectively, which indicated that removal of RBCs already largely occurred in the first hour after transfusion. The mean 24-h PTR of 3-day-, 10-day- and 21-day-stored RBCs was 89.8%, 81.9% and 76.7%, respectively, and they all complied with the gold standard of RBC viability. The fraction that was removed in the first 24 hours after transfusion was probably composed of irreversibly damaged and/or damage-susceptible RBCs. The damage to the RBCs might have occurred throughout that entire period from the moment of collection until transfusion [15]. A longer storage period was prone to cause more damaging insults, which would explain why a much larger fraction of RBCs with long storage period perishes in the first 24 hours. The T50 and the MPL of 3-day-, 10-day- and 21-day-stored RBCs that had survived the first 24 hours were not significantly different. Refrigerated storage of RBCs might slow the aging process of the RBCs up to a certain stage, and past this stage they would be removed in the first 24 hours after transfusion. We also determined p50 and effective oxygen-carrying amount, and found that p50 and effective oxygen-carrying amount of RBCs both decreased with prolongation of the preservation period. The decrease in oxygen-delivering capacity of stored RBCs with prolongation of the storage period was primarily due to the decrease of 2,3-DPG in the stored blood [16]. Preventing or recovering the decrease of 2,3-DPG would contribute to maintaining the quality of preserved RBCs. Furthermore, we obtained the correlation between the effective oxygen-carrying amount and storage time by regression analysis. Then we further investigated the relationship between survival and the effective oxygen-carrying amount, and found the different RBC suspensions with same total effective oxygen-carrying amount had nearly identical number of survival cells that can circulate in the body after transfusion. This result implied that appropriate increment transfusion with old stored RBCs might play the same role as the transfusion with the fresh RBCs. Recently, several studies reported that the fraction, which is removed in the first 24 hours after transfusion is primarily responsible for transfusion side effects, especially in transfusion-dependent patients [23, 24]. In this study, the transfusion side effects were not observed in all patients. Therefore, we propose that transfusion with old stored RBCs will be cost-effective in the transfusion-independent patients.

In summary, this study demonstrates that the PTR of RBCs decreased with storage time increasing, and the 24-h PTR of 3-day-, 10-day-, and 21-day-stored RBCs complied with the gold standard of RBC viability. We found

that both p50 and effective oxygen-carrying amount of RBCs decreased with storage time increasing *in vitro*. In addition, we further investigated the relationship between survival and the effective oxygen-carrying amount, and found the different RBC suspensions with same total effective oxygen-carrying amount had nearly identical number of survival cells that can circulate in the body after transfusion. These findings will provide theoretical basis and practical direction for scientific and efficient blood transfusion.

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Disclosure of conflict of interest

None.

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