Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/mjafi

Original Article

Comparative study of automated cryopreservation of red blood cells

Col Alok Sen^{a,*}, Brig A. Khetarpal (Retd)^b^a Commanding Officer, Armed Forces Transfusion Centre, New Delhi 110010, India^b Ex Commanding Officer, Armed Forces Transfusion Centre, New Delhi 110010, India

ARTICLE INFO

Article history:

Received 30 May 2012

Accepted 23 June 2013

Available online 24 September 2013

Keywords:

Automated red cell cryopreservation
ACP 215

Frozen blood

ABSTRACT

Background: Strategic blood reserves are an important component in meeting blood needs and this can be accomplished through the establishment of a frozen blood program.**Methods:** One hundred units of packed RBC were glycerolized using the Haemonetics ACP 215 automated cell processor and placed in a -86°C deep freezer for freezing and storage. Product weight, hematocrit, RBC count, WBC count and hemoglobin were recorded prior to freezing. Twenty five bags were thawed and deglycerolized after every three months starting at one year from the date of first glycerolization. In addition to the earlier parameters the bags were assessed for supernatant osmolality, pH, supernatant hemoglobin, ATP levels and supernatant potassium and from these red cell recovery, percentage hemolysis, supernatant glycerol and red cell viability were estimated. All tests were repeated at the end of 7 and 14 days.**Results:** The mean red cell recovery was found to be 86.12% on Day 0 and 84% on Day 14. All the bags showed residual glycerol and pH within the acceptable limits upto Day 14. Percentage hemolysis, Mean ATP levels and mean supernatant potassium levels were within acceptable limits upto Day 14. All the units were sterile upto Day 14.**Conclusion:** The data in this study showed that the red cells which were glycerolized using the automated platform ACP 215, frozen at -80°C for more than a year and deglycerolized again using the ACP 215 had excellent viability while being stored at 4°C during the 14 days of post-thaw storage.

© 2013, Armed Forces Medical Services (AFMS). All rights reserved.

Introduction

India has a population of 1.21 billion and supports nearly one-sixth of the world's population. As per the latest estimates annual requirement of blood across the country is about 10,000,000 units against an annual collection of approximately 8,500,000 units of blood resulting in a discrepancy between

supply and demand. Strategic blood reserves are an important component in meeting blood needs and this can be accomplished through the establishment of a frozen blood program. The concept of a frozen blood reserve is twofold: to freeze excess units of blood during times of surplus for use during a time of shortage and to freeze units of rare blood types for use later by patients with special transfusion needs.

* Corresponding author.

E-mail address: aloksen19@yahoo.com (A. Sen).

0377-1237/\$ – see front matter © 2013, Armed Forces Medical Services (AFMS). All rights reserved.

<http://dx.doi.org/10.1016/j.mjafi.2013.06.005>

Freezing RBC with glycerol as a cryoprotectant dates back to 1950.¹ After several modifications, two methods employing glycerol at a concentration of 20% and 40% (W/V) termed Low Glycerol Method (LGM) and High Glycerol Method (HGM) respectively are still in use.^{2,3} The method using 20% glycerol requires storage at -196°C under liquid nitrogen, whereas the method using 40% glycerol requires frozen storage at -80°C . Both these methods require post-thaw washing to reduce glycerol concentration to less than 1%.³ Frozen red cells are FDA approved for storage at -80°C or below for 10 years. Post deglycerolization the RBCs can be stored at 4°C for no more than 24 h if open system for deglycerolization is used due to the potential for bacterial contamination.⁴ Because of this short shelf life, frozen blood programs have not been widely adopted worldwide.

The Haemonetics ACP 215 is an automated, functionally closed system for the glycerolization and deglycerolization of human RBCs that uses a disposable single use kit having inline $0.22\ \mu$ filters to deliver solutions, a disposable polycarbonate bowl with a disposable seal and an integrally attached shaker and printer to record the process. The system freezes and thaws blood with automated technology that improves operational logistics and cost effectiveness. More importantly, the new thawing process extends the shelf life of the thawed blood to 14 days. These enhancements make it easier for blood collectors and hospitals to implement or expand frozen blood reserves. The red cells processed by closed system are sterile after deglycerolization and the prolonged post-thaw life of the red cells greatly enhances their flexibility as a red cell product, since they can be shipped to hospitals and utilized with the same inventory control procedures as other red cell products. In times of emergency or blood shortages, frozen red cells could provide a realistic backup blood supply.

Various parameters have been studied across the globe before cryopreservation and in the post-thaw blood. The important ones were pH, osmolality, RBC volume and plasma hemoglobin. Various studies have also been carried out on the viability of cells after prolonged periods of cryopreservation.^{5,6} Studies have also been done by using preservatives for prolonging life of blood after thawing.^{6–8}

Military and civilian blood collectors around the world are considering frozen blood programs. Frozen red cells were first used by the US in Vietnam. RBCs frozen by the high glycerol method and stored at -80°C were deglycerolized manually and used within hours of deglycerolization. However, the manual method was so resource intensive that only 465 units were used over 180 days i.e. 2.5 units per day at a time when the US military usage in Vietnam averaged 600 units per day.⁹ During the cold war US military analysts envisioned the requirement of upto 160,000 units of blood per day in case of war and this revived the interest in frozen blood. Approximately 1% of the blood collected in the U.S. is frozen each year, and most of this frozen blood – around 100,000 units – is held by the military for war efforts rather than by civilian collectors. The Italian military has also recently implemented a new frozen blood program using the ACP 215 automated cell processor.

The aim of this project was to standardize and evaluate an automated system of cryopreservation of red cells using an automated cell processor to glycerolize and deglycerolize red

cells which is capable of producing cryopreserved red cells having a 14 day post-thaw life and to assess the quality of the packed red cells prior to glycerolization, immediate post deglycerolization and during the 14 day post-thaw period. The final objective was to assess the feasibility of development of a frozen blood bank at this center for availability of blood of rare blood groups for unexpected high demands as a back up to liquid blood for operational requirements and during disaster situations.

Material and methods

A red cell glycerolization – deglycerolization project was undertaken w.e.f 17 Dec 07 to 07 Dec 09.

Glycerolization

One hundred healthy male voluntary donors who met the requirements for acceptable blood donors donated one unit of whole blood comprising of 450 ml. All the donors had a body weight of $>60\ \text{kg}$ and hematocrit of $>40\%$. Two pilot samples were taken from the blood bag after collection. One of these was sent to the immunohaematology lab for confirmation of ABO/Rh using Biorad ID Card “DiaClon ABO/D + Reverse Grouping” and screening for the presence of red cell alloantibodies using a 3 cell screening panel (Biorad ID DiaCell I-II-III Asia). The other sample was sent to the infectious markers lab for screening for HIV, Hepatitis B, Hepatitis C, Syphilis and Malaria.

The CPDA-1 whole blood was centrifuged at a speed of 2400 rpm (1913 g) for 7 min at 22°C , and the plasma removed to produce a hematocrit of $75 \pm 5\%$. The volume of RBCs collected was between 180 and 220 ml per unit. The RBCs were stored at 4°C before being taken up for glycerolization and freezing using the Haemonetics ACP 215 automated cell processor within 5 days of blood collection. On the 4th/5th day the red cell bag was removed from the blood bank refrigerator and brought to a temperature range of 20°C (68°F) to 30°C (86°F) by placing in a plasma thawer bath at 37°C . The bags were then connected to a 1000 ml bag appropriate for freezing using a sterile connecting device and the red cell concentrate was transferred into the 1000 ml bag. The disposable glycerolization set was then connected to the 1000 ml PVC bag using the sterile connecting device and glycerol (57%) W/V solution was pumped through the disposable glycerolization set into 1000 ml PVC red cell freezing bag using the Haemonetics Automated Cell Processor (ACP 215) during glycerolization process to achieve a final glycerol concentration of 40% W/V glycerol. Glycerolized red cell bags were then centrifuged at 1940 rpm (1248 g) for 10 min at 22°C with brake setting at zero. The unit was then carefully removed from the centrifuge and placed on a plasma extractor. All the visible supernatant was then expressed to remove supernatant glycerol and prepare a glycerolized red blood cell concentrate with a hematocrit value of $60 \pm 5\%$. The bag was then removed from the plasma extractor and the glycerolized red cells were resuspended and mixed thoroughly by manual agitation. The product weight, hematocrit, RBC count, WBC count and hemoglobin were estimated and from these the product volume, absolute RBC

volume and the total RBC count of the bag were calculated. One sample from the bag was sent for aerobic, anaerobic and fungal culture. This bag was then placed in rigid cardboard boxes and stored in a -86°C deep freezer. As per FDA approved protocol no more than 4 h were allowed to lapse between the time the red cells were removed from the 4°C refrigerator and the time they were placed in the -86°C deep freezer. The total time taken for the process of glycerolization was 20–30 min.

Deglycerolization

Twenty five bags were thawed and deglycerolized after every three months using the ACP 215 system starting at one year from the date of first glycerolization i.e. at one year, one year three months, one year six months and one year nine months post glycerolization. The process of deglycerolization was as follows:

Thawing

The units of glycerolized frozen red cells were first thawed by placement into one of the pouches of a plasma thawer at 36°C for 35 min. To achieve the 14 day post-thaw life of the red cells it is imperative that the processing must be completed and the deglycerolized red cells must be placed in a $2-6^{\circ}\text{C}$ refrigerator within 2 h of removal from the -86°C deep freezer. Once thawing was complete the bags were removed from the plasma thawer and checked for any breaks by gently compressing the unit.

Deglycerolization

After thawing, the plastic freezing bag was secured to the platform on the Haemonetics ACP 215 by means of magnetic mounting posts. Once the 'START' key was pressed, the ACP 215 first evaluated the integrity of the sterile connection weld by holding and monitoring a negative pressure. The ACP 215 then automatically started the deglycerolization procedure. During the deglycerolization procedure the machine first added 50 ml of 12% sodium chloride (hypertonic) solution to the red cell bag. The hypertonic saline shocked the red cells causing the glycerol to come out of the cells. Next the machine added the first dilution of 340 ml 0.9% sodium chloride – 0.2 g % glucose solution to the thawed red blood cells and then stopped for an equilibration delay of 60 s. The 0.9% sodium chloride – 0.2 g% glucose solution has two roles – it washes away the glycerol and at the same time rehydrates the red cells. A total of five dilutions/washes were done by the machine. At the end of the fifth cycle the ACP 215 transferred 240 ml of AS-3 additive solution into the bowl. AS-3 (Additive Solution – 3) was the additive preservative solution which gave the red cells a 14 day post-thaw life. AS – 3 contains 1100 mg of Dextrose, 30 mg Adenine, 276 mg Monobasic sodium phosphate, 410 mg Sodium chloride, 588 mg Sodium citrate & 42 mg of Citric acid per 100 ml. The color of the effluent fluid in the effluent line was then compared to the color of the Haemonetics plastic color comparator. If the color was less than 5, the hemoglobin level was acceptable the procedure was complete.

In addition to all the earlier parameters (product weight, product hematocrit, product RBC count, WBC count and

product hemoglobin), certain additional tests were carried out on the deglycerolized bags like supernatant osmolality, pH, supernatant hemoglobin, ATP levels and supernatant potassium and from these the red cell recovery, percentage hemolysis, supernatant glycerol and red cell viability were estimated. The supernatant osmolality was estimated using the Freezing point Osmometry method while the supernatant hemoglobin was estimated using the HemoCue Plasma/Low Hb System. All tests were repeated on the post-thaw samples at the end of 7 days and 14 days in order to assess the quality of red cells during and at the end of the 14 day post-thaw period. Samples from the bags were sent for culture on Day 0 and Day 14 to assess the sterility. The total time taken for the process of deglycerolization was 40 min.

Results

A total of 99 units of glycerolized red cells were successfully prepared. One unit was discarded due to the blood bag leakage during centrifugation. All 99 bags were successfully deglycerolized. The product quality details of the glycerolized and deglycerolized red cells are summarized in Table 1.

The mean product volume of the glycerolized bags was 222.60 ml with a mean hematocrit of 75.49% resulting in a mean absolute red cell volume of 168.04 ml. All the bags were sterile on aerobic, anaerobic and fungal culture and negative for red cell alloantibodies and infectious markers screening in the samples collected on Day 0 post deglycerolization & on Day 14 after storage for 14 days at 4°C . The mean product volume of the deglycerolized bags on Day 0 of deglycerolization was 317.69 ml with a mean hematocrit of 47.13% resulting in a mean absolute red cell volume of 149.73 ml. The product hematocrit after deglycerolization (47.13%) in this study appears to be below the mandatory requirement of 51–53% as per AABB. However, this was probably due to the fact that approximately 20–30 ml of blood was removed from the glycerolized bags prior to freezing for carrying out the various assays and cultures and since the deglycerolization procedure was done on an automated platform the entire volume of AS-3 required for preservation (240 ml) was automatically added to the bags without taking the initial volume reduction into consideration. The resultant dilution caused a drop in the hematocrit and made it appear to be below the mandatory requirement. In a routine procedure this would not have happened since the entire volume of blood would have been there in the bag.

Total red cell count & red cell recovery

The mean total red cell count was estimated in the glycerolized bags before freezing by multiplying the RBC count with the product volume and was found to be 1,818,642 cells. After thawing and deglycerolization, the mean total red cell count was again estimated in the deglycerolized bags on Day 0, Day 7 & Day 14. The mean red cell recovery in the deglycerolized bags was then calculated and was found to be 86.12% on Day 0 which was much higher than the mandatory requirement of minimum 80% red cell recovery.¹⁰ The recovery was 79.89% on Day 7 and 78.13% on day 14. However, this reduction in red cell

Table 1 – Product details of glycerolized and deglycerolized red cells.

Glyc	Unit age	Net product wt (g)	Product vol (ml)	Product hct (%)	Absolute RBC vol (ml)	RBC count (×10 ⁶)	Total RBC count	WBC count (/ μ l)
Deglyc								
Glyc		235.96	222.60	75.49	168.04	8.17	1,818,642.00	9743.67
Deglyc	Day 0	336.75	317.69	47.13	149.73	4.93	1,566,211.70	196.23
Deglyc	Day 7	318.85	300.80	47.09	141.65	4.83	1,452,864.00	32.71
Deglyc	Day 14	312.49	294.80	46.29	136.46	4.82	1,420,936.00	32.54
Glyc	Unit age	Supernatant vol (ml)	Product Hgb (g/dl)	Supernatant Osmolality (mosm/kg)	Product pH (37 °C)	Supernatant Hgb (g/dl)	Hemolysis (%)	Pre-Glyc Total RBC Count
Deglyc								
Glyc		54.56	24.42					
Deglyc	Day 0	167.96	14.71	323.27	6.65	0.05	0.18	1,818,642.00
Deglyc	Day 7	159.15	14.70	326.07	6.67	0.14	0.50	1,818,642.00
Deglyc	Day 14	158.34	14.68	331.61	6.63	0.24	0.88	1,818,642.00
Glyc	Unit age	RBC Recovery (%)	ATP (mmol/gHb)	Supernatant K+ (mmol/L)	Sterility	Antibody Screen	Infectious Markers	
Deglyc								
Glyc					Sterile	Negative	Negative	
Deglyc	Day 0	86.12	3.93	2.52	Sterile			
Deglyc	Day 7	79.89	3.98	25.55				
Deglyc	Day 14	78.13	2.71	34.06	Sterile			

recovery was due to the blood samples that were removed from the bags on Day 0 & Day 7. The red cell recovery was found to be more than 84% both on day 7 & day 14 once the volume removed was adjusted.

Post glycerolization and post deglycerolization WBC count

The pre-glycerolization WBC count was estimated using the Sysmex KX 21 hematology auto analyzer and the mean pre-glycerolization WBC count was found to be 9743.67/ μl resulting in a mean total WBC content of 2168.94×10^6 WBC per unit. The post deglycerolization WBC count was estimated at Day 0, Day 7 & Day 14 using the Nageotte Chamber and the mean post glycerolization WBC count was found to be 196.23/ μl on Day 0, 36.71/ μl on Day 7 & 36.54/ μl on Day 14 resulting in a mean total WBC content of 62.34×10^6 WBC per unit on Day 0, 9.83×10^6 WBC per unit on Day 7 & 9.59×10^6 WBC per unit on Day 14. These results were very similar to those observed by Valeri et al in a study in 2001⁸ and meant that the freezing, thawing and washing resulted in an end product that was log 2 to log 3 leucodepleted without the use of any leucodepletion filters.

Supernatant osmolality

In the red cell freezing process, the cells are protected by glycerol during freezing and storage at -80°C . The glycerol must be removed from the frozen & thawed red blood cells prior to transfusion since residual glycerol of greater than 1% may produce hemolysis of the red blood cells following transfusion. Quality control testing for the adequacy of deglycerolization is done by measuring the supernatant osmolality of the deglycerolized red blood cells. Supernatant osmolality of <400 mosm/kg H_2O indicates a residual glycerol concentration of 1% or less.¹¹ In our study, the mean

supernatant osmolality was found to be 323.27 mosm/kg H_2O on Day 0, 326.07 mosm/kg H_2O on Day 7 & 331.61 mosm/kg H_2O on day 14 with none of the bags showing a supernatant osmolality of >400 mosm/kg H_2O on any day. Thus all the bags showed a residual glycerol of less than 1 % post deglycerolization.

Product pH

The mean product pH was 6.65 on Day 0, 6.67 on Day 7 & 6.63 on Day 14 which was well within the acceptable limits for stored red cells. Normal packed red cells stored in CPDA-1 have a mean pH of 6.71 on day 35 while those stored in AS3 have a mean pH of 6.5 on day 42.¹² A pH over 6.6 correlates with better storage of red cells.¹³ In this study, all the bags showed a pH over 6.6 upto Day 14.

Supernatant hemoglobin & percentage hemolysis

The total hemoglobin of the packed cells and the supernatant hemoglobin in the deglycerolized units were estimated in order to determine the percentage hemolysis. The percentage hemolysis was calculated from the formula:

$$\% \text{ Hemolysis} = \frac{\text{Supernatant Hb} \times \text{Supernatant Vol}}{\text{Total Hb} \times \text{Total Vol}} \times 100$$

The mean supernatant hemoglobin was 50 mg/dl (0.05 g/dl) on Day 0, 140 mg/dl (0.14 g/dl) on Day 7 & 240 mg/dl (0.24 g/dl) on Day 14. Taking the supernatant volume, total hemoglobin & total vol of the bags into account the percentage hemolysis was found to be 0.18% on Day 0, 0.50 % on Day 7 & 0.88% on Day 14. This was well within the acceptable limits of 1% upto the last day of assessment.^{12,13} Earlier studies have shown the one percent red cell lysis is approximately equivalent to a mean hemoglobin level of 250 mg/dl.⁵

ATP levels & red cell metabolism

While the actual mechanism of the loss of viability of stored red blood cells is uncertain, a commonly held view is that primarily this is due to a loss of adenosine triphosphate (A.T.P.) in the cell, and that this produces a block in the metabolism of glucose. In the 1950s, it was first noted that red cells that had lost their adenosine triphosphate (ATP) did not survive well during storage.¹⁴ In this study we tried to evaluate the viability and metabolism of the red cells by estimating their ATP content. Earlier studies have shown that the mean ATP value corresponds to 3.5–4.56 $\mu\text{mol/g Hb}$.¹³ With storage the ATP levels in red cells gradually decrease and come down to upto 45% (i.e. approximately 1.6–2.0 $\mu\text{mol/g Hb}$) by day 35 in CPDA-1 preserved red cells and upto 59% (i.e. approximately 2.0–2.7 $\mu\text{mol/g Hb}$) by day 42 in AS3 preserved red cells.¹² In this study the mean ATP levels were found to be 3.93 $\mu\text{mol/g Hb}$ on Day 0, 3.98 $\mu\text{mol/g Hb}$ on Day 7 & 2.71 $\mu\text{mol/g Hb}$ on Day 14. This was well within the acceptable limits upto the last day of assessment indicating a good viability in the red cells upto 14 days post deglycerolization. The results also reconfirmed the initial increase in ATP content in stored red cells that had been observed in several previous studies.^{15,16}

Supernatant potassium

Red cells lose potassium and gain sodium during storage. This is because the $\text{Na}^+ - \text{K}^+$ gradient is normally maintained by a $\text{Na}^+ - \text{K}^+$ ATPase that does not function well at 4 °C and because of increased red cell permeability due to damage to the cells. This increased extracellular potassium is usually not significant except in neonatal transfusions. Normal CPDA-1 preserved blood shows plasma potassium levels of upto 27.3 mmol/L in whole blood and 78.5 mmol/L in packed cells by day 35 while AS3 preserved blood shows plasma potassium levels of upto 46 mmol/L by day 42. In this study the mean supernatant potassium levels were found to be 2.52 mmol/L on Day 0 post deglycerolization, 25.55 mmol/L on Day 7 & 34.06 mmol/L on Day 14. This was well within the acceptable limits upto the last day of assessment again indicating a good viability in the red cells upto 14 days post deglycerolization.

Discussion

This study represented the first Red Cell Automated glycerolization project conducted in India using the Haemonetics ACP 215 system. This is a milestone for the frozen blood program in India. The data in this study showed that the red cells which were glycerolized using the automated platform ACP 215, frozen at –80 °C for more than a year and deglycerolized again using the ACP 215 had excellent viability while being stored at 4 °C during the 14 days of post-thaw storage. This conclusion is based on the various parameters discussed above in comparison to the results of previous studies where similar data produced good viabilities. Thus the study was successful in carrying out its two aims viz. standardizing and evaluating the automated system of cryopreservation of red cells using the ACP 215 automated cell processor to glycerolize and deglycerolize red cells capable of producing red cells

having a 14 day post-thaw life; and assessing the quality of the packed red cells prior to glycerolization, immediate post deglycerolization and at the middle and end of the 14 day post-thaw period.

In recent years, India has experienced localized blood shortages particularly during times when an adequate number of donors are not able to donate due to sickness (like in flu season), weather and holiday and vacation schedules. Shortages also occur due to unanticipated demand from particular patients, accidents, natural disasters, and crises. On the other extreme, mass blood donations following natural or man-made disasters sometimes results in overstocking of blood in central blood banks since people are unsure of whether or not the local blood supplies were sufficient. As a result, sometimes blood is in such oversupply in the months immediately following the disaster that some of it has to be discarded resulting in a tragic waste of a valuable resource. These tragic events make it clear that Indian blood banks particularly the Armed Forces must have a blood reserves in place in case of local and national emergency as well as acts of war.

Freezing red cells enables us to overcome their short life span and make long-term storage possible. In addition to looking after the transfusion needs during natural and man-made calamities, frozen red cells can also cater for the requirement of rare blood groups particularly in emergencies & encourage the cause of autologous blood donation since the blood can be stored for as long as 10 years. The biggest problem with autologous blood donation is that the donor knows that if the blood is not used immediately i.e. within 5 weeks, it is of no use to him. However, if autologous blood is frozen it can be made available to the donor for as long as 10 years after donation. This would be a great incentive for the donor to go for autologous blood donation and would in turn encourage autologous blood donation in India which is presently negligible.

Across the world several studies and trials have been and are being carried out to find and develop blood substitutes. However, none of the trials have been successful so far and in the absence of any viable blood substitutes a frozen blood program is the only option for augmenting the current liquid blood stores particularly for disasters and operational scenario. The biggest problems associated with freezing blood was that deglycerolizing the frozen blood manually was extremely labor intensive requiring a full day to process a single bag and the end of it the red cells had a post-thaw outdate of only 24 h. However, with the ACP 215 automated system for glycerolization freezing and subsequent deglycerolization of red cells both these problems are addressed adequately. As seen from our study, a single operator on a single machine can comfortably deglycerolize 8–10 bags in a day which then have a post-thaw life of upto 14 days when stored at 4 °C. This presents an important development in cryopreservation and the establishment of a frozen blood bank for the Armed Forces to augmenting the existing liquid blood supply.

Conflicts of interest

All authors have none to declare.

REFERENCES

1. Smith AU. Prevention of hemolysis during freezing and thawing of red blood cells. *Lancet*. 1950;2:910.
2. Valeri CR, Valeri DA, Anastasi J, et al. Freezing in the primary polyvinyl chloride plastic collection bag: a new system for preparing and freezing non-rejuvenated and rejuvenated red blood cells. *Transfusion*. 1981;21:138–149.
3. Meryman HT, Hornblower M. A method for freezing and washing red blood cells using a high glycerol concentration. *Transfusion*. 1972;12:145–155.
4. *Guide to the Preparation, Use and Quality Assurance of Blood Components*. 7th ed. Strasbourg: Council of Europe; 2001.
5. Valeri CR, Ragno G, Pivacek LE, et al. An experiment with glycerol frozen red blood cells stored at -80°C for up to 37 years. *Vox Sang*. 2000;79:168–174.
6. Myhre BA, Marcus CS. The extension of 4°C storage time of frozen – thawed red cells. *Transfusion*. 1992;32:344–348.
7. Valeri CR, Pivacek LE, Cassidy GP, et al. Post transfusion survival (24 hour) and hemolysis of previously frozen deglycerolized RBCs after storage at 4°C for upto 14 days in sodium chloride alone or sodium chloride supplemented with additive solutions. *Transfusion*. 2000;40:1337–1340.
8. Valeri CR, Ragno G, Pivacek LE, et al. A multicenter study of in vitro and in vivo parameters of human red blood cells frozen with 40% W/V glycerol and stored after deglycerolization for 15 days at 4°C in As-3: assessment of RBC processing in the Haemonetics Model 215. *Transfusion*. 2001;41(7):933–939.
9. Neel S. *Medical Support of the US Army in Vietnam '65–70*. Washington DC: Department of the Army; 1973:155.
10. American Association of Blood Banks (AABB). *Standards for Blood Banks and Transfusion Services*. 26th ed.; Bethesda. 2009.
11. Valeri CR. *Blood Banking and the Use of Frozen Blood Products*. Boca Raton: CRC Press; 1976.
12. Brecher ME. *Technical Manual*. 15th ed. Bethesda, MD: American Association of Blood Banks; 2005:673.
13. Klein Harvey G, Anstee David J. *Mollison's Blood Transfusion in Clinical Medicine*. 11th ed. Oxford: Blackwell Scientific Publications; 2005:369.
14. Gabrio BW, Finch CA, Huennekens FM. Erythrocyte preservation: a topic in molecular biochemistry. *Blood*. 1956;11:103–113.
15. Meryman HT, Hornblower M, Syring RL. Prolonged storage of red cells stored at 4°C . *Transfusion*. 1986;26:500–505.
16. Meryman HT, Hornblower M, Syring R, Mesbankarimi N, Bross J. Refrigerated storage of washed red cells. *Vox Sang*. 1991;60:88–98.