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Review Article

Fifty (50) Year Experience at the NBRL, Boston, Massachusetts to Study the Survival and Function of Fresh, Liquid, and Freeze Preserved Human and Baboon Red Blood Cells, Platelets, and Plasma Clotting Proteins

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Abstract

This article reports what occurred and what was learned during the 50 years when the Naval Blood Research Laboratory (NBRL) was supported by the U.S. Navy's Research and Development Command, the Office of Naval Research, and the Congress of the United States to study the survival and function of frozen RBC, frozen platelets, and frozen plasma clotting proteins obtained from healthy volunteers, patients, and baboons.

Keywords: Platelets; Red blood cells; Plasma clotting proteins

Introduction

Between 1965 and 1974, the NBRL was located at the Chelsea Naval Hospital which was renamed the Boston Naval Hospital. Two books were written to report the studies performed and the data reported during this period: Blood Banking and the Use of Frozen Blood Products and Hypovolemic Anemia of Trauma: The Missing Blood Syndrome. In addition a monograph published in 2006 reported the 30-year experience at the NBRL to study the survival and function of fresh, liquid preserved, and frozen baboon RBC, platelets, and plasma clotting proteins: The survival and function of baboon red blood cells, platelets and plasma proteins: a review of the experience from 1972 to 2002 at the Naval Blood Research Laboratory, Boston, Massachusetts [1]. In the 65 peer-reviewed NBRL publications reported in the monograph, the bleeding time measurement was shown to be an accurate method to assess the hemostatic function of fresh, liquid preserved and cryopreserved baboon red blood cells, platelets, and plasma clotting proteins.

In 2007, the monograph: Non-surgical bleeding diathesis in anemic thrombocytopenic patients: Role of temperature, RBC, platelets, and plasma clotting proteins [2] was published. This monograph reports that nonsurgical blood loss correlated to the bleeding time in normal volunteers and in patients. The bleeding time measurement was affected by the temperature and the function of the RBC, platelets, and plasma clotting proteins. The monograph summarized 45 peer reviewed NBRL publications which measured the bleeding time in normal volunteers and patients to assess the hemostatic effect of the red blood cells, platelets, and plasma proteins to reduce the bleeding time and reduce the nonsurgical blood loss. The monograph identified that the temperature had a significant effect on the bleeding time. Hypothermia produced a significant increase in the bleeding time and increase in nonsurgical blood loss and increase in temperature reduced the bleeding time and reduced the nonsurgical blood loss.

In 2013 the book: Forty-five years of research at the NBRL, Boston, Massachusetts: Chaotic observations, serendipity, and patience was published.

This book reports the current procedures to freeze human red blood cells, platelets, and plasma clotting proteins. The clinical results using frozen platelets to treat patients following cardiopulmonary bypass surgical procedures and the clinical results in patients subjected to traumatic injuries in war zones treated with frozen RBC, frozen platelets, and frozen plasma by the Netherlands military are reported.

Universal donor group O Rh positive and group O Rh negative RBC, group O single donor leukoreduced platelets resuspended in AB plasma, and AB plasma eliminate the need to provide fresh whole blood to treat patients subjected to traumatic injuries in war zones. The current method to freeze single donor leukoreduced platelets containing 2.5 to 3.0 X 10^{11} platelets equivalent to the number of platelets isolated from 4 to 6 units of fresh whole blood treated with 5% DMSO in 0.9% NaCl, the supernatant DMSO removed prior to freezing and storage in a -80°C mechanical freezer, and following thawing resuspended in a unit of AB plasma eliminate the need to provide the platelets in fresh whole blood and single donor leukoreduced apheresed platelets stored at room temperature for 5 days with agitation.

Freezing at 2-3°C per minute by storage of human platelets treated with 6% DMSO in a -80°C mechanical freezer produces a bimodal population of platelets. One population of platelets that circulates and the other population of platelets that functions to reduce non-surgical bleeding. In studies performed in the baboon, autologous platelets frozen with 6% DMSO, thawed, washed and resuspended in autologous plasma produced a population of GpIb normal and reduced factor V binding baboon platelets with an *in vivo* recovery of 48% and a linear lifespan of less than 6 days. The other population of GpIb reduced and increased factor V binding baboon platelets was rapidly removed from the circulation within 5 minutes and was associated with a reduction in nonsurgical blood loss [3].

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The FDA requires that preserved RBC have 24-hour post transfusion survival of 75%, However, they make no regulations requiring the function of the RBC immediately or shortly after transfusion. One should not assume that because the survival of preserved RBC and preserved platelets is satisfactory, their ability to function following transfusion will also be satisfactory. The FDA also assumes that when a sick patient receives 25% nonviable compatible RBC with a transfusion, the recipient will suffer no adverse effects. Although there are no data to show how many nonviable compatible RBC can be safely administered to sick patients, it is known that if a patient receives 10 units of RBC with a 24-hour posttransfusion survival value of 75%, then 2.5 units of the RBC will be nonviable. The removal of these compatible nonviable RBC by the Reticuloendothelial (RE) system may interfere with the removal of infectious disease agents, tumor cells, particulate matter present in the stored RBC, and could affect cellular and humoral immunity.

The FDA considers preserved platelets to be acceptable if the *in vivo* recovery is 66% that of fresh platelets and the lifespan is 50% that of fresh platelets but again offers no guidelines with regard to their hemostatic function. Although in vitro levels of RBC ATP, DPG and p50 levels give an indication of the *in vivo* function of the preserved RBC, there are no in vitro tests that can predict the *in vivo* function of fresh and preserved platelets. Studies at the NBRL have shown that when preserved platelets function satisfactorily they will correct an aspirin induced prolonged bleeding time in normal volunteers and in healthy baboons [2,3].

Liquid preserved autologous platelets that were stored at room temperature (22+2°C) with agitation for up to 48 hours were found to have acceptable *in vivo* recovery and survival and were able to function to reduce an aspirin-induced prolonged bleeding time in normal volunteers [2-6]. On the other hand, when autologous baboon platelets were stored at room temperature for 3 or 5 days, they did circulate but did not reduce the prolonged bleeding time in aspirin treated healthy baboons [7].

Between 1988 and 1992, the NBRL collaborated with Shukri Khuri, Chief of Surgery and Chief of Cardiothoracic Surgery at the West Roxbury Veteran's Administration Hospital, to evaluate liquid preserved and washed previously frozen platelets transfused to Cardiopulmonary Bypass (CPB) patients [6]. The previously frozen platelets had been frozen with 6% DMSO at 2-3°C per minute and stored at -80°C for a mean of 289+193 days (SD) and for as long as 2 years and were washed prior to transfusion. A prospective randomized study was conducted in 73 patients undergoing CPB surgery. The study was designed to measure nonsurgical blood loss in 53 patients, twenty (20) patients were excluded because of surgical blood loss, i.e. blood loss not related to the surgical procedure and not controlled by surgical intervention after neutralization of the heparin with protamine sulfate. Nonsurgical blood loss was collected intraoperatively and during the 24-hour postoperative period.

The allogeneic single donor washed previously frozen platelets transfused to these patients had been processed in the following manner. The platelets were frozen at the University of Massachusetts in Worcester, Massachusetts, and transported in the frozen state with dry ice to the NBRL where they were stored in -80°C mechanical freezers for at least 3 months. They were then transported in the frozen state in insulated containers with dry ice to West Roxbury Veteran's Administration Hospital blood bank where they were stored at -80°C in mechanical freezers. After thawing, the platelets were washed and stored in ACD plasma at room temperature without agitation for as long as 5 hours. The platelets were transfused to the patients after the CPB surgery. Twenty-nine (29) patients received liquid preserved platelets and 24 patients received the frozen platelets.

The prospective randomized study compared the need for allogeneic RBCs and Fresh Frozen Plasma (FFP) to treat the nonsurgical blood loss in the two groups of patients. The patients who received previously frozen washed platelets showed a reduction in the nonsurgical blood loss and required fewer units of allogeneic RBC and FFP than the patients who received liquid preserved platelets stored with agitation at 22°C for a mean of 3.4 days. The platelet survival 2 hours after transfusion was 37% for patients who received the liquid preserved platelets compared to 24% for the patients who received the previously frozen washed platelets [6].

The total number of liquid preserved platelets infused was $6.9X10^{11}+3.9X10^{11}$ per patient which was significantly greater than the $4.5X10^{11}+2.1X10^{11}$ per patient for the previously frozen washed platelets. The difference was the result of the in vitro loss of platelets in which 70% of the platelets were recovered following the freeze-thaw-wash procedure. The *in vivo* recovery and function of the liquid preserved and cryopreserved platelets in these patients were similar to values seen in studies in which liquid preserved and cryopreserved platelets were transfused to aspirin-treated human volunteers and baboons. Although in this study, the *in vivo* recovery values were higher for the liquid preserved allogeneic platelets than for the washed previously frozen platelets, nonsurgical blood loss was lower in the patients who received the washed previously frozen platelets and they required fewer units of allogeneic RBCs and FFP [6].

In the paper in Transfusion [8] Bilgin YM and associates reported the postoperative complications associated with transfusion of platelets and plasma in cardiac surgery. The authors reported that to improve hemostasis, platelets and Fresh Frozen Plasma (FFP) are often transfused in the perioperative and postoperative period, however, neither the efficacy nor the safety of platelets and plasma transfusions have been demonstrated. The authors failed to identify that the paper written by Khuri SF and associates described above compared the effects of cryopreserved and liquid preserved platelets on hemostasis and blood loss after cardiopulmonary bypass [6]. The prospective randomized study compared the need for liquid preserved red blood cells stored at 4 C and fresh frozen plasma stored at -20°C to treat the nonsurgical blood loss in the two groups of patients. Although the *in vivo* recovery values of the liquid preserved platelets were higher than those of the previously frozen washed platelets, the patients who received the washed previously frozen platelets had significantly reduced nonsurgical blood loss and required fewer units of liquid preserved red blood cells and fresh frozen plasma than the patients who received liquid preserved platelets stored at room temperature for a mean of 3.4 days with agitation. No adverse effects were observed in either group [6-8].

In 2000 the NBRL modified the method of freezing platelets with 6% DMSO at 2-3°C per minute by storage in a -80°C mechanical freezer. Similar to the method our laboratory used to simplify RBC

freezing by removing the supernatant glycerol prior to freezing, the NBRL simplified freezing of platelets by removal of the supernatant DMSO prior to freezing. Removal of the supernatant DMSO removes about 95% of the DMSO and eliminates the need for post-thaw washing prior to transfusion [9]. With the modified method the previously frozen platelets did not require washing prior to transfusion. When platelets are washed the in vitro recovery is reduced by 25%. With the modified method, the thawed platelets are not washed but are diluted with 10 ml to 20 ml of 0.9% NaCl and stored without agitation at room temperature for 6 hours. The Freeze-Thaw (FT) recovery is approximately 90%, with 5-8% platelet microparticles, *in vivo* recovery is 25-30%, and the linear lifespan is 7 days. The diluted platelets have a bimodal population: one population is GPIb-normal and annexin V-reduced and the other is GPIb-reduced with increased annexin V binding.

Lelkens CCM and associates have reported the use by the Netherlands military of: Group O Rh positive and group O Rh negative RBC frozen with 40% W/V glycerol and stored at -80ºC for at least 10 years; group O single donor leukoreduced platelets frozen with 5% DMSO with removal of the supernatant DMSO solution before freezing and storage at -80ºC for at least 2 years and following thawing resuspended in AB plasma; and AB plasma stored at -30ºC and then at -80ºC for at least 10 years to treat patients who required these blood products [10-12].

Lelkens CCM and associates experience in the Netherlands military have demonstrated for the first time that frozen RBC, frozen platelets and frozen plasma stored at -80ºC can be used to treat patients without the need for a "walking blood bank" and fresh whole blood [13-15]. Henkelman S and G Rakhorst in Transfusion [16] have similarly reported the safety and therapeutic effectiveness of frozen RBC, frozen platelets, and frozen plasma stored in -80ºC mechanical freezers to treat combat casualties without the need for fresh whole blood.

The past 50 years have seen many significant improvements in cryopreservation procedures. Removing supernatant glycerol from RBC and DMSO from platelets prior to freezing have simplified the post thaw processing. Platelets diluted with 10 ml to 20 ml of 0.9% NaCl after thawing can be stored at room temperature without agitation for 6 hours prior to use. With the functionally closed Haemonetics ACP215 instrument, deglycerolized RBC can be stored at 4 C in AS-3 (Nutricel) for 2 weeks. With this instrument, the volume of solution needed to deglycerolize RBC is now only 2.0 liters compared to 3.2 liters with the Haemonetics Blood Processor 115 and 6.8 liters with the Huggins cytoglomerator. Mechanical freezers maintained at -80ºC are needed for the storage of these safe and therapeutically effective frozen blood products for use by both the military and civilian communities to supplement the liquid preservation of RBC stored at 4ºC, platelets stored at 22ºC with agitation, and fresh frozen plasma and cryoprecipitate stored at -20ºC. The -80ºC mechanical freezer contains a dual-cascade air-cooled compressor and is attached to a carbon dioxide tank to be triggered to add the liquid carbon dioxide when the temperature decreases to -65ºC because of electrical or mechanical failures can be deployed in combat areas by the military. Frozen blood products consisting of RBC, platelets, and plasma can be transported using dry ice in insulated containers which maintain the temperature of -65ºC to -80ºC as documented by the experience of the U.S. Navy during the Vietnam War in 1968 to 1974 and the Netherlands military in the Middle East combat zones in Iraq, Afghanistan, and Bosnia in 2000 to 2012.

The Netherlands military has been actively freezing universal donor group O Rh positive and group O Rh negative RBC, single donor leukoreduced frozen group O platelets and frozen AB plasma in -80 C mechanical freezers. These frozen blood products have been collected from donors meeting FDA regulations that were safely transported and stored in -80ºC mechanical freezers without breakage. These blood products have been obtained from screened blood donors and tested for the mandated infectious markers prior to freezing and have eliminated the need for fresh whole blood or apheresed leukoreduced platelets stored at room temperature for 5 days with agitation to treat patients suffering traumatic injuries with therapeutically effective outcomes and without adverse events.

Dr. John Badloe at the ATACCC meeting on August 16, 2010 at St. Pete, Florida reported that in Afghanistan from 2000 to 2010, 859 patients received 6,335 blood products which include 1918 units of frozen red blood cells, 841 units of liquid preserved red blood cells, 2560 units of frozen plasma and 1,016 units of frozen platelets with no transfusion reactions. Fresh whole blood was not used by the Netherlands military because the fresh whole blood could not be tested prior to transfusion for the mandated infectious disease markers whereas all the frozen blood products were tested for the mandated infectious disease markers prior to freezing from the donors who were screened prior to donation.

Dr. Badloe reported the Netherlands military experience in the Middle East war zones using frozen blood products, i.e. frozen group O Rh positive and group O Rh negative RBC, frozen AB plasma, and frozen group O single donor leukoreduced platelets with removal of supernatant DMSO, all frozen and stored at -80ºC in mechanical freezers at ratio of 1:1:1 increased survival of patients from 44% to 84%. No adverse events were reported and only frozen blood products which were safe, available, effective and efficient in the treatment of patients requiring at least 10 units of red blood cells in a 24-hour period for resuscitation were used without the need for fresh whole blood.

An abstract which was reported by Dr. John Badloe and Dr. Femke Noorman from the Ministry of Defense, Military Blood Bank, Leiden, Netherland at the annual meeting of the American Association of Blood Banks (AABB), San Diego, CA October 22-25, 2011 confirms the procedures provided by the NBRL, Boston, MA to freeze human RBC, plasma, and platelets in -80ºC mechanical freezers. This report by the Netherlands military demonstrates that fresh whole blood advocated by Colonel William Crosby and the U.S. Army is no longer used by the Netherlands military and the fresh whole blood can be replaced by frozen RBC, frozen plasma, and frozen platelets with significantly improved survival of the massively transfused patients [14]. Dr. Femke Noorman and Dr. John Badloe and associates also presented one oral presentation and three [3] poster presentations on the use of frozen blood products at the AABB annual meeting from October 6-9, 2012 in Boston, MA [15].

The utilization of the -80ºC mechanical freezers to freeze RBC,

platelets, and plasma by the Netherlands military has demonstrated the safety and therapeutic effectiveness of these frozen blood products without the need for fresh whole blood or apheresed platelets to treat military and civilian casualties requiring more than 10 units of red blood cells within a 24-hour period in combat zones in Afghanistan and Iraq. It is of interest that the Netherlands investigators pioneered the use of liquid nitrogen to freeze blood products but now utilize -80°C mechanical freezers to freeze red blood cells, platelets and plasma to treat combat casualties.

Studies can now be done to compare the safety and therapeutic effectiveness of universal donor RBC frozen for at least 10 years, group O platelets frozen for at least 2 years, and AB plasma frozen for at least 10 years (all blood products frozen and stored at -80°C with a range of -65°C to -90°C) without the need for fresh whole blood to the current use of fresh whole blood, liquid preserved RBC stored at 4°C in additive solutions for as long as 42 days, single donor leukoreduced apheresed platelets stored with agitation at room temperature for 5 days, and fresh frozen plasma and cryoprecipitate stored at -20°C for one year on mortality and morbidity in the recipients.

For the past 50 years the Naval Blood Research Laboratory, Boston, MA has been supported by the U.S. Navy to study the safety and therapeutic effectiveness of frozen RBC, frozen platelets and frozen plasma to treat wounded casualties. Human RBC treated with 40% W/V glycerol with the removal of the supernatant glycerol prior to freezing at -80°C for 10 years, thawed, deglycerolized using the Haemonetics Blood Processor ACP215 instrument and stored in AS-3 at 4 C for 2 weeks; single donor group O leukoreduced platelets treated with 6% DMSO, concentrated to remove the supernatant DMSO prior to freezing of the platelets in a -80°C mechanical freezer for at least 2 years, thawed, diluted with 0.9% NaCl and stored at room temperature for 6 hours, and group AB plasma frozen at -80°C for at least 10 years, thawed and stored at 4 C for 24 hours are now available to treat wounded casualties.

For the past 15 years frozen group O Rh positive and group O Rh negative RBC; frozen single donor leukoreduced group O platelets concentrated to remove the supernatant DMSO prior to freezing with 5% DMSO at -80°C and, after thawing, resuspended in AB plasma; and frozen AB plasma have been used by the Netherlands military to treat wounded casualties. Using these blood products, the Netherlands military in combat zones in Afghanistan, Iraq, and Bosnia has reported significant improvement in the survival of wounded casualties who required more than 10 units of red blood cells in a 24 hour period from 44% to 84%. The blood products were transfused at a ratio of 4 units of frozen group O red blood cells, 3 units of frozen AB plasma, and one unit of single donor group O leukoreduced platelets containing 2.5 to 3.0 X 10^{11} platelets with no adverse effects.

The recent paper by Henkelman S and Rakhorst G "Does modern combat still need fresh whole blood transfusions?" reported that the Dutch military blood bank eliminated the use of fresh whole blood on site and implemented the routine use of frozen group O Rh positive and group O Rh negative deglycerolized RBC, frozen single donor leukoreduced group O platelets, and frozen AB plasma to treat wounded casualties in war zones [16]. The RBC, platelets, and plasma are frozen and stored at -80°C in mechanical freezers. Following thawing, the deglycerolized RBC are stored at 4°C in the additive solution AS-3 for 14 days, the thawed AB plasma stored at 4 C for 7 days, and the thawed single donor leukoreduced platelets containing 2.5 to 3.0X10^{11} platelets in AB plasma stored at room temperature for 6 hours.

A comparison between the U.S. Army procedures and the Netherlands procedures to provide blood products to treat combat casualties needs to be performed to determine the logistic requirements, outdating of blood products, compatibility, cost of transportation, availability, safety and therapeutic effectiveness. The quality and quantity of the blood products to resuscitate casualties requiring at least 10 units of red blood cells in a 24-hour period and the mortality and morbidity utilizing the U.S. Army procedures compared to the Netherlands procedures in combat zones need to be determined. The Netherlands military has documented that -80°C frozen red blood cells, plasma and platelets were deployable, available, compatible, safe and effective in the treatment of trauma patients with or without massive blood loss in military theatre.

In the study to compare the procedures utilized by the Netherlands military to the current FDA approved procedures to provide blood products; the volume and composition of the resuscitation fluids used with the blood products need to be reported. The morbidity and mortality associated with the current FDA approved blood products can be compared to the use of frozen RBC, frozen platelets, and frozen plasma without the need for fresh whole blood and apheresed platelets which Netherlands military has successfully utilized to treat combat casualties in war zones from August 2006 to February 2012; 2,175 units of frozen RBC, 1,070 units of frozen platelets, 3,001 units of frozen plasma and 879 units of liquid preserved RBC stored at 4 C were transfused to 1,011 casualties without any transfusion reactions observed. The blood products were obtained from pre-screened donors and the blood products were tested for infectious diseases prior to freezing [15].

For the past 15 years, the Netherlands military have utilized universal donor RBC, platelets and plasma all frozen and stored at -80°C in a mechanical freezer to treat wounded casualties with excellent clinical results and without adverse events. The data reported by the NBRL over the past 50 years and the Netherlands military over the past 15 years now support that the Food and Drug Administration, the American Red Cross, Health and Human Service, and the Department of Defense should recommend the use of universal donor frozen group O positive and O negative RBC, frozen group O leukoreduced platelets and frozen AB plasma. All the blood products frozen and stored at -80°C provide safe and therapeutic effective blood products to treat patients. The frozen blood products will eliminate or reduce the severe adverse events of mortality and morbidity associated with the current FDA approved red blood cell products, platelet products and plasma products. The universal donor group O positive and O negative frozen RBC, group O leukoreduced single donor frozen platelets, and AB frozen plasma obtained from male donors will provide blood products that provide acceptable *in vivo* survival and function to treat patients and reduce or eliminate the severe adverse events associated with the current FDA approved blood products.

In the response to the paper "Transfusion of older stored blood

and risk of death" published by Wang D and associates published in Transfusion [17] an editorial was written by Warkentin TE and Eikelboom JW titled "Old blood bad? Either the biggest issue in transfusion medicine or a nonevent" [18]. These authors stated "the demonstration of an association (if indeed there is one) between the age of transfused blood and outcome would be useless if one could not do anything about it". This editorial failed to acknowledge that freeze preservation of red blood cells, platelets and plasma has been reported to provide safe and therapeutically effective blood products from 2006 to 2012 to successfully treat 1,011 civilian and military casualties in war zones in Iraq and Afghanistan by the Netherlands military using -80°C mechanical freezers to freeze and store universal donor group O Rh positive and group O Rh negative red blood cells, single donor leukoreduced group O platelets, and group AB plasma without the need for fresh whole blood and apheresed platelets.

It is imperative that civilian and military blood banking communities change their methods of collection and preservation of blood products if they are really interested in providing patients with the safest and most therapeutically effective blood products and in avoiding risks associated with transfusion.

The Food and Drug Administration (FDA), Health and Human Services (HHS), the American Red Cross (ARC) and the blood banking community have focused on further testing of blood products to reduce the rate of disease transmission as well as disinfection of red blood cells, platelets, and plasma. More importantly, they should be looking at the quality of the blood products being transfused. We know that the length of storage of blood products affects their survival and function and that transfusion of nonviable compatible RBC, antibodies to granulocytes and WBC HLA antigens, and biologically active substances affect the patient's clinical outcome. One of the easiest ways to prevent the severe adverse effects that have been observed is to ensure that the transfused blood products survive and function at an optimum level and that the levels of antibodies to granulocytes and WBC HLA antigens and biologically active substances are reduced. The best way to ensure this is to store liquid preserved human red blood cells at 4°C in additive solutions for no more than 2 weeks and leukoreduced platelets at room temperature for no more than 2 days. These liquid preserved blood products can be used in conjunction with frozen RBC, frozen platelets and frozen plasma all stored in -80°C mechanical freezer.

The reluctance of the blood banking community to consider any changes that could improve the safety and therapeutic effectiveness of blood products brings to mind two very relevant quotations: one by Maurice Maeterlinck is that "At every crossway on the road that leads to the future, every progressive spirit is opposed by a thousand men appointed to guard the past". The other by Winston Churchill is that "Occasionally man will stumble on the truth but will manage to pick himself up and continue on as if nothing had happened". It is time now to investigate the current blood banking procedures and to seek ways to improve them.

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