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FINAL REPORT

RESEARCH IN HUMAN BLOOD PRESERVATION BY FREEZING - INCLUDING PHASE A) STUDIES ON REDUCTION OF INCIDENCE OF POSTTRANSFUSION HEPATITIS IN BLOOD SO PRESERVED AND PHASE B) THE DEVELOPMENT OF DISPOSABLE PLASTIC EQUIPMENT TO SO PRESERVE BLOOD

Phase A: Published under authors - J. L. Tullis, J. Hinman, M. T. Sproul, R. J. Nickerson

Incidence of Posttransfusion Hepatitis in Previously Frozen Blood, JAMA 214:719-723, 1970.

Phase B: Disposable Plastic Centrifuge Bowls in the Separation of Red Cells and Plasma and in the Processing of Frozen Blood

by

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Prepared for publication in TRANSFUSION

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13. ABSTRACT Phase A: Incidence of Posttransfusion Hepatitis in Previously Frozen Post-transfusion hepatitis was significantly reduced by a transmembrane Blood washing procedure. The Blood Research Institute method constitutes mechanical centrifugation, high glycerol concentration, slow freeze and thaw, and a 4-liter washing procedure for deglycerolization. Four cases of histologically proved hepatitis (one icteric, three anicteric) occurred in a control group of 104 recipients receiving 442 transfusions (median 3) of cells resuspended in autologous plasma, an incidence of 9 per 1,000. No hepatitis occurred in the treatment group receiving 623 transfusions (median 4) of cells resuspended in albumin ($p = 0.029$). The mechanism by which hepatitis virus is eluted or inactivated is not revealed by the study. It appears that all or sufficient virus is physically displaced from the cell so that they are non-infective. Glycerolization, freezing, long-term storage and thawing have nothing to do with loss of infectivity as supported by the transmission of hepatitis virus to four recipients of cells resuspended in plasma. The study does not reveal information on aspects of host resistance, viral induction or dose to infectivity relationships as a function of plasma resuspension. The decrease in hepatitis by the Blood Research Institute transmembrane washing procedure of previously frozen blood cannot be interpreted to imply: a) that other transmembrane washing methods or b) that extracellular (saline) washing of fresh or frozen blood or c) that use of packed red cells will result in similar loss of infectivity.

(continued page 3)

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Phase A:

Blood Research Institute transmembrane washing procedure

transmembrane washing procedure on thawed deglycerolized previously frozen red blood cells

Post-transfusion hepatitis, reduction of by Elution of hepatitis virus

Phase B:

Simplified method for automated RBC transmembrane washing

Simplified method for freezing, deglycerolization, plasma and red cell separation

Disposable plastic centrifuge bowls
(Haemonetics Corporation
266 Second Avenue
Waltham, Mass. 02154)

Latham Processor
(Cryogenic Technology, Inc.
266 Second Avenue
Waltham, Mass. 02154)

Phase B

ABSTRACT

A simplified method for automated red cell processing is described. The method uses sterile, disposable plastic centrifuge bowls with attached tubing and reagent solutions. It can be used for the separation of red cells and plasma and the glycerolization and deglycerolization of red cells for storage in the frozen state. Data is presented on operational performance of the equipment and on red cell yields and post-thaw transfusion survival. (Manuscript attached)

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DISPOSABLE PLASTIC CENTRIFUGE BOWLS IN THE SEPARATION OF RED CELLS AND PLASMA AND IN THE PROCESSING OF FROZEN BLOOD

ABSTRACT

A simplified method for automated red cell processing is described. The method uses sterile, disposable plastic centrifuge bowls with attached tubing and reagent solutions. It can be used for the separation of red cells and plasma and the glycerolization and deglycerolization of red cells for storage in the frozen state. Data is presented on operational performance of the equipment and on red cell yields and post-thaw transfusion survival.

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This laboratory has been concerned for two decades with the design of centrifuge equipment for the sterile separation and washing of blood cells and for the automation of red cell glycerolization and deglycerolization techniques. Three generations of centrifuge bowls have been developed, each embodying the principle of cell separation at low G force while blood flows through the centrifugal field. The first device, the Cohn Fractionator (1), was developed primarily as a research tool. It could be adapted to the preparation of concentrates of platelets, red cells, and buffy coat, and to the instillation and removal of glycerol in connection with red cell freezing. Although the Cohn equipment proved highly useful in the development of such blood processing techniques (2) (3) (4) (5), it was not intended as a routine tool for standard blood bank use. Accordingly, a second series of simplified bowls, with the same geometric design were developed in our laboratories in collaboration with the Arthur D. Little Company. Data obtained with this equipment proved essentially similar to the Cohn system (6) (7). The third step was the development of disposable, plastic centrifuge bowls which could be inserted into a similar drive mechanism for the routine application of cell processing methods to blood banks and hospital services. It is the purpose of this report to describe experience with such new disposable bowls for plasma-red cell separation and for glycerolization, freezing, and deglycerolization of red cells. A separate report will describe the disposable, plastic, platelet processing bowls.

The study was conducted in four phases: 1) Preliminary testing of 50 bowls for operational performance, sterility, and red cell recovery; 2) Complete in-vitro studies of 15 units of blood collected in ACD solution and then glycerolized, frozen, thawed, and deglycerolized; 3) Combined in-vitro and in-vivo study of 10 additional units of blood glycerolized, frozen, thawed, deglycerolized and labled with 51 Cr and 125 I and transfused autologously; 4) Clinical data on 42 additional units of thawed deglycerolized cells reconstituted to 550 ml volume and transfused into routine hospitalized patients at the New England Deaconess Hospital.

METHODS

Blood was collected in Abbott ACD bags and glycerolized at room temperature, unless processing was delayed for intervals from 30 minutes to 2 hours, in which case, it was stored at 4 C. until the time of glycerolization.

The percent cell recovery in the freezing studies was computed as follows: The volume of blood used was calculated by 1) weighing the original bag of ACD blood, 2) subtracting the weight of the empty bag and 3) dividing the net weight by 1.050. The volume was then multiplied by the gram % hemoglobin obtained from the original bag for the total hemoglobin value. Blood loss (red cells remaining in the bags, lines, bowls, effluent wash, plasma, glycerol and deglycerolized solutions) was then computed by lysis and subsequent measurement of total hemoglobin present. The percent loss was obtained by dividing the gram % loss by the 100% value; and

the percent recovery by subtracting the % loss from 100.

Red cell, white cell and platelet counts were done by standard techniques. Hematocrit values were measured in capillary tubes in a micro hematocrit centrifuge. Red cell indices were calculated from red cell count, hemoglobin and hematocrit. Whole blood hemoglobin was measured by the cyanmethemoglobin method (8) and the plasma hemoglobin by the benzidine method (9).

Sedimentation at 60 minutes was carried out by an accelerated dextran method (10). Red cell densities were measured in pthalate esters (11). Osmotic fragilities were performed in the Danon fragiligraph (12).

Glycerol assays were performed by the ultraviolet method of the Boehringer Mannheim Corporation (15).

The pH of whole blood and plasma was measured at 37 C. with a capillary system and radiometer..

Glucose, ATP and 2,3 DPG were measured by the enzymatic methods of Keitt using enzymes from Boehringer-Mannheim (16). Sodium and potassium concentrations were measured with a Baird flame photometer. Protien concentration was assayed by the biuret method.

Cultures were inoculated on thioglycolate, Saboraud's medium and blood agar plates, incubated at 37 C., 20 C. and 4 C. by standard methods.

Post-transfusion survival was measured by the method of Gibson et al (13), employing 125 I for plasma volume and 51 Cr for red cell labelling. The total blood volume was calculated from the plasma volume and hematocrit value, the red cell volume being the

difference between total and plasma volume. The 100% retention value was obtained by dividing the total 51 Cr given by the red cell volume and this was used as a reference for calculation of the percent survival of red cells from samples taken at 10, 20, and 30 minutes after starting the transfusion and of all subsequent samples. These values were plotted on semi-log paper and a fitted line extrapolated to time zero, the intercept being taken as the percent immediate posttransfusion survival. Intensive evaluation of this technique has shown a standard error of ± 5%.

The forms used for patient informed consent, blood processing, bacteriologic culture and in-vitro records were of standard type. Calculation of posttransfusion survival was as previously reported (13).

The glycerol freezing method and solutions were those described by Tullis and associates (3) (4) (6) (14). This system embodies the following principles: 1) centrifugal separation of plasma and red cells followed without interruption by sequential glycerolization to a high intra cellular concentration (40% ± 5%); 2) slow freezing (approximately 6 hours to each ambient temperature); 3) storage at -80 C. (± 15 C.) in mechanical refrigeraters; 4) thawing by 15 minutes of immersion in a 37 C. water bath; 5) degylcerolization by attachment to the same type of sterile, disposable centrifuge bowl and introduction of a decreasing gradient of glycerol in the presence of lactate solutions, washing with buffered electrolyte solution and reintroduction of the red cells into the thawed autologous plasma (reconstituted whole blood) or into albumin (red cells in albumin) or into an empty bag (packed red cells in saline).

All solutions for glycerolization and deglycerolization and all plastic harnesses for attachment of the bags of blood or red cells to the sterile bowls were prepared by either Cutter Laboratories or Abbott Laboratories. The formulas are listed in table 1. For all studies, the plastic centrifuge bowls were inserted into an automated, Model 10 processor* especially constructed for use with this equipment. A Rustrack recorder and thermocouple Model 133B were used for all temperature recordings.

Assembly of Harnesses and Bowls:

1) The carton, containing the pre-selected harness, was fully opened, inspected and all side clamps closed. A disposable bowl package was then removed from its container, and connected to the harness, with appropriate sterile techinque. The bottle of glycerolizing or deglycerolizing solution were next aseptically connected to the harness followed by a unit of whole blood for glycerolization, or a unit of thawed cells for deglycerolization. The assembly of blood and solutions were placed inverted onto a portable rack and transferred to the processor.

2) Glycerolization:

Following the insertion of the proper programmer code into the drive mechanism, the cycle button was activated. Plasma automatically was separated, and the red cells glycerolized. The only manual operation needed was closure of a pinch clamp on the plasma bag port and opening of the waste bag port during operation. At the end of 24 minutes, the glycerolized red cells were pumped into the red cell bag; the bowl was washed with 50 ml of 50% glycerol and the wash added to

* Cryogenic , Inc., Waltham, Massachusetts.

the cells. The bag of cells was detached, mixed, and a sample withdrawn from an attached diverticulum of the bag for sterility and glycerol assay. The plasma bag was detached and three samples withdrawn from the inlet tube; a 4-inch sealed section was taken for sterility check. The plasma bag was wrapped in aluminum foil and placed in a pasteboard carton with the cells and stored at -80 C. The tubing leading from the ACD bag was sealed with blood for subsequent sampling. The empty ACD bag was weighed and recorded, the volume of wash waste was measured, and a sample taken for hemoglobin assay.

3) Deglycerolization:

Twenty-five units of frozen cells and plasma were thawed in a 37 C. water bath for 15 minutes. After pre-run procedure, the thawed cells were attached to appropriate harness and automatically washed with 4000 ml of deglycerolization solutions. At the end of the cycle (25 minutes or less), the cells were displaced into the red cell bag, the bad detached and mixed. Samples were withdrawn for sterility and glycerol assay. The original plasma was then added sterily to the washed cells, except for a 25 ml aliquot retained for experimental analysis, sterility check and gram stain. The entire contents of the sealed bag of reconstituted blood was used for chemical and cytological studies in the 15 units not administered autologously. The volume of wash waste was measured and a sample taken for hemoglobin assay. The residual blood in lines and the bowl was lysed with water, the volume measured, and a sample taken for hemoglobin assay. In the units administered in-vivo, a sterile 50 ml aliquot was removed for labelling and transfusion and the

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balance was used for chemical and cytological study. In the 42 units used for homologous transfusion, only a small aliqout was removed for cross-march and sterility study.

4) Storage:

Storage was in mechanical Harris refrigerators* at -80 C. (+ 15 C.) for periods of 1-3 months before use. It was the purpose of this study only to compare the processing effectiveness of the present equipment with that of the previous slow freeze method, studies of which have been previously reported.

RESULTS

I. General

A total of 147 disposable bowls was evaluated. The clarity, configuration, freedom from flow limitations, attachment to and release from the Rotor were good. The first 25 bowls were used for pressure testing and temperature checks. The remaining 122 were used under sterile conditions. One unit of blood was lost due to bowl failure due to malocclusion of the concentricity centering knob of the bowl in the processor which was used. The concentricity centering knob of the bowl proved to be undersized for the Rotor, and this subsequently was altered.

The harness functioned well, and although some were kinked when removed from the package, they were free from flow limitations. One unit of blood was lost due to a pin hole leak along the seal of the bag.

*Model 17 L-2.

The model 10 Processor, used to drive the centrifuge shaft and automated pumping motors, performed flawlessly. There was minor variation of house current which affected the programmer causing variations in flow rate on occasional units. This variation in current and irregular edges of the first program film caused an estimated loss of 4% when compared with previous studies using manual control.

II. Operational Performance

A. Temperature Changes

(Equipment and Empty Bowls): The two temperature ends of two thermocouples were placed in a constant 20 C. water bath. The distal ends of the thermocouples were plugged into a Rustrak Recorder whose temperature range was 10 C. to 25 C. The thermocouple readings were identical. Temperature checks were then made at the bottom, middle, and top of the model 10 housing with the cover on, with and without a disposable plastic centrifuge bowl for 55 minutes of continuous running. The maximum temperature rise was 1 C.

(Solutions flowing through Disposable Bowls): The calibrated thermocouples were placed in thin stainless steel thermocouple wells. One well was attached to the inflow port of the disposable bowl, the other to the outflow port of the bowl. Both were positioned as close to the rotary seal as possible. Complete glycerolization studies were carried out on 25 disposable bowls. Twenty-five simulated glycerolization and deglycerolization studies were performed using water rather than blood. The maximum temperature rise between inflow

and outflow during "glycerolization" was 3 C., and during "deglycerolization", 1 C. The 3 C. rise during "glycerolization" was during the 15 minutes of slow-flow rate of 15 ml per minute. The disposable bowl temperature checks were identical with control studies using the reusable stainless steel bowl. Twenty-five standard glycerolizations were then performed with blood while the temperature of inflow and outflow was recorded. Similar constancy of temperature was recorded.

B. Bacteria

No positive cultures were obtained at 4 C., room temperature, or 37 C. in the thioglycolate broth passed through the bowl in 25 simulated runs or in Sabouraud's media at room temperature, and no positive cultures were obtained in the multiple samples removed form the 15 in-vitro blood studies, 10 in-vivo studies or 42 units administered as routine transfusions after deglycerolization.

A deliberately contaminated bowl was connected to sterile harness and solutions. A simulated run was then made to demonstrate the adequacy of the sterility culture method. Each sample resulted in a positive culture at 37 C., 4 C., and room temperature.

III Characteristics of Whole Blood, Cells and Plasma Processed in the Equipment

A. Whole Blood Prior to Processing

Samples from the bags of anticoagulated whole blood following collection were tested for control values. These showed the expected range commonly encountered. The values are shown in Tables 3+5 for: red blood cells, white blood cells, platelet count, hematocrit, hemoglobin, dextran sedimentation rate, red cell density, red cell osmotic fragility, pH, glucose, ATP, 2,3 DPG, sodium potassium, supernatant hemoglobin, and intracellular potassium.

B. Plasma Recovery and Content

The characteristics of the separated plasma are also detailed in Table 3.

C. Glycerolized Red Cells

The effect of glycerolization on red cell size and shape is shown in Table 2. In general, there was an increase in MCV following glycerolization. The partition of blood cells at different steps of the processing and the effects of red cell density, osmotic stability and sedimentation characteristics are shown in Table 3.

Preliminary experience with the disposable equipment showed that mixing in the new bowl was less complete than for the stainless steel prototype previously reported (6). This was believed due to the lighter weight, improved balance, and hence less turbulent rotation of the centrifuge bowls.

Levels of final glycerol concentration using our previously reported solution volume, flow rate and operating conditions, varied from 20 gms % to 43 gms % with a mean of 34 gms %. Although there is no a priori reason for anticipating less satisfactory stabliity of the red cells from this lower glycerol content than achieved in our previous reports (6) (14), it was found that a simple acceleration of solution flow rates by setting the programmer so as to complete the entire process in 24 minutes rather than 32 provided enough agitation of cells in the revolving bowl to provide good mixing. The range of final glycerol content of the treated cells by this method (now standard) was 33.5 to 49.3 with a mean of 38.5 gms %.

D. Deglycerolized Cells

The effect of combined glycerolization and deglycerolization on cell size is shown in Table 2. There was a further increase in cell volume after deglycerolization, but this returned to 94.3 after resuspension of the washed cells in their original ACD plasma. The effect of glycerolization and deglycerolization on the metabolic characteristics of the red cells is shown in Table 4. Only nominal decreases in glucose in the deglycerolized cells were those expected after washing with solutions free of added sugar. The level of glycerol achieved in the washed, thawed cells varies from .150 gms % to .450 gms % with a mean of .309 gms % when using the slow 33-minutes deglycerolization time developed for our previous studies. Acceleration of the program cycle to 25 minutes again resulted in

good agitation of the cells in the revolving bowl with improved mixing and glycerol removal. The final glycerol content in 10 studies varied from .041 - 0.21 with a mean of 0.139 gms % in the washed packed cells and from .028 to 0.145 with a mean of .095 gms % in the reconstituted whole blood at its full volume of 550 ml.

E. Reconstituted Blood

The in-vitro analyses of the washed, deglycerolized red cells after resuspension in their autologous plasma and ready for tagging or transfusion are shown in Table 2 and 3. The plasma and cells were from all standpoints suitable for transfusion purposes. The final recovery of red cells was 89.19 % (Table 5).

F. In-vivo Red Cell Survival

The experimental data for 10 units of deglycerolized cells labled with 125 I and 51 Cr and given as autologous transfusion are given in Table 6. The mean posttransfusion survival was 91.0 %. This included 2 units (AB 64 and AB 61) which were shipped by Air Express a distance of 2,000 miles in the frozen state to demonstrate the red cell stablilty while glycerolized. One unit #65 was discarded and replaced (65 A). Although it appeared normal at the time of deglycerolization, a fracture of the bag had occurred from trauma during shipment in dry ice.

G. Post-thaw Stability

A previously demonstrated superiority of the high glycerol,

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