

# **COMPOSITIONS AND METHODS FOR THE STORAGE OF RED BLOOD CELLS**

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## **STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT**

### **BACKGROUND OF THE INVENTION**

The invention generally relates to compositions and methods associated with the storage of red blood cells (RBCs). In particular, it relates to an improved RBC storage composition and processes and applications thereof.

The ability to store and preserve red blood cells (RBCs) for later re-infusion into patients is a relatively recent technological development that was the harbinger to modern surgical practice. Such preservation is scientifically tricky and the steps to achieving longer storage duration and higher quality re-infused red blood cells have been incremental. As soon as they are collected from a donor, red blood cells begin to die as they coagulate, starve, lose ATP, 2,3-DPG, membrane surface area and integrity, and hemoglobin (Hb). Rous & Turner in 1916 and Robertson in 1917 first demonstrated successful whole blood storage. Acid-citrate-dextrose (ACD, 1943), comprising citrate as an anti-coagulant and dextrose as the sole nutrient utilized by red blood cells, and Citrate-phosphate-dextrose solution (CPD, 1957), adding phosphate as a metabolic source and for membrane retention, were subsequently approved for 21-day storage of whole blood. CPD with adenine (CPDA-1, 1979) was later introduced and used for extending the shelf life of stored whole blood and packed RBCs for up to 5 weeks.

Initially, storage compositions were designed to be acidic to prevent the caramelization of the glucose during the heat sterilization performed in the final production step. In the 1950s, adenine was discovered to be useful as an additive and replaces the adenine lost by deamination. In the 1970s it became desirable to remove the plasma from the collected whole blood for platelets and for the manufacture of plasma derivatives. This, however, led to a reduction in the percent recovery of the resulting "packed RBC."

To circumvent this, compositions known in the art as additive solutions (AS) were developed to restore volume, nutrients, and other useful RBC stabilizers. Additive solution compositions for the preservation of red blood cells (RBCs) after their separation from whole blood are intended to be tailored specifically to the needs of RBCs. The development of certain

additive solutions extended RBC storage to 6 weeks in 1981. Red blood cells (RBCs) stored in these solutions, however, undergo steady deterioration after 6 weeks as determined by the inability of 75% of such cells to survive in the circulation for 24 hours after re-infusion back into the human donor. It has been observed that during continued refrigerated storage, glucose is consumed at a decreasing rate, as the concentration of metabolic waste, i.e. lactic acid and hydrogen ions, increases. Such a decrease in the rate of glucose metabolism leads to depletion of adenosine triphosphate (ATP), which directly correlates to the recovery of RBCs when the cells are returned to the circulation. Additive solutions such as Adsol.RTM (AS-1), Nutricel RTM (AS-3), Optisol RTM (AS-5), and ErythroSol RTM were designed to extend the storage of RBCs at 1-6° C. All three ASs currently licensed in the U.S., AS-1, AS-3, and AS-5, contain saline, adenine, glucose and some citrate and/or mannitol as "membrane protectants." AS-3 also contains monosodium phosphate. Each of the U.S.-licensed ASs meet the licensure requirements for 6-week RBC storage, but fail to achieve 7-week storage. Presently licensed RBC additive solution compositions were developed before the RBC storage lesion (defined herein as the sum of the survival- and/or function-limiting effects of storage on RBCs) was understood to be an apoptotic process.

Almost all of the whole blood collected now is made into components, and the RBC fraction is stored as packed RBCs. For blood drawn into the additive solution systems, RBCs are packed by centrifugation, plasma is removed so that RBCs make up 80% of the volume, and then 100 ml of additive solution is added sterily. The resulting suspensions have a RBC volume fraction of approximately 55%. RBCs stored in the conventional FDA-approved additive solutions can be stored for only 6 weeks with an acceptable 24-hour in vivo recovery.

To increase the time of acceptable in vivo recovery of RBCs re-infused into patients after a storage period, attempts have been made to improve the additive solutions and storage processes. In "Studies In Red Blood Cell Preservation-7. In vivo and in vitro Studies With A Modified Phosphate-Ammonium Additive Solution," by Greenwalt et al., Vox. Sang. 65:87-94 (1993), the authors determined that an experimental additive solution (designated EAS-2) containing (in mM): 20  $\text{NH}_4\text{Cl}$ , 30  $\text{Na}_2\text{HPO}_4$ , 2 adenine, 110 dextrose, 55 mannitol, formulated at a pH of 7.15, is useful in extending the storage shelf-life of human RBCs from the current standard of 5-6 weeks to an improved standard of 8-9 weeks. However, packed RBCs stored in EAS-2 were not directly infusible but required the removal of the supernatant with a washing step prior to transfusion due to the presence of ammonium in the additive solution.

In "Studies in Red Blood Cell Preservation-8; Liquid Storage of Red Cells in Glycerol-Containing Additive Solution," *Vox. Sang.* 67:139-143 (1994), Greenwalt et al. described an additive solution (designated EAS-25) that allowed 73 percent recovery of packed red cells after nine weeks. However, the resulting RBC units contained 1 percent glycerol and thus, are not safe for transfusion in humans in large amounts.

In "Extending the Storage of Red Cells at 4.degree. C.," *Transfus. Sci.* 15:105-115 (1994) by Meryman et al., acceptable viability of RBCs stored in very dilute suspensions at low hematocrit for as long as 27 weeks were demonstrated. However, such stored RBC suspensions were not acceptable for direct infusion due to their high content of potassium and ammonia and their low volume fraction of RBCs. The 5 L of solution for 200 mL of RBC required to produce his observed beneficial effects were not clinically practicable.

With respect to approved and commercially available products, the additive solutions presently licensed in the U.S. work for only 6 weeks with an average recovery of 80%. Two additive solutions presently licensed in Europe work for 7 weeks with an average recoveries of 77% (ErythroSol from Baxter Healthcare, La Chatre, France) and 75% (PAGGS mannitol from Maco Pharma). Novel solutions recently described by Kurup et al. (*Vox Sang* 2003: 85:253-261) may be expected to have shorter storage times because of the lower ATP concentrations.

In response to the deficiencies in these prior findings, the present inventors developed lower volume disodium phosphate-containing alkaline experimental additive solutions (EASs) that partially neutralize the effect of collecting blood into acidic anticoagulant solutions such as CPD (citrate-phosphate-dextrose), and showed that these EASs improved RBC ATP concentrations, reduced hemolysis, and appeared to decrease RBC membrane morphological changes and loss (see U.S. Patent Nos. 6,150,085 and 6,447,987 to Hess and Greenwalt). Various EASs were shown to support between 9 and 12 weeks of storage. Although these EASs yielded superior performance results, they contained sodium chloride and were formulated to require a relatively large volume resulting in greater dilution of the stored RBC, thus increasing the risk of hemodilution in multiply transfused patient recipients. In addition, the presence of sodium chloride created a solubility limit on the amount of buffering salts and phosphates that the system could sustain at desirable volumes.

Increased duration of RBC storage remains an important consideration during periods when demand is high but intermittent, such as during wartime, and for geographical regions that require transfusable blood but only on an inconsistent and sporadic basis. In fact, given the

current level of reported waste due to expiration of the safe storage period prior to realization of a demand in general, increasing the duration of time that RBCs may be safely stored is an ongoing ubiquitous concern.

Thus, there is a need for RBC storage compositions formulated to retain or enhance recovery and performance benefits in the lower volumes of conventional additive solutions. There is a continuing need in the blood storage and transfusion art for improved RBC storage that results in longer storage duration, better recovery percentage, and improved physiological functioning of the transfused RBC. Consequently, there remains a need for improved RBC storage compositions and processes of manufacture thereof. There is also a continuing need for additive compositions which allow the RBC suspension to which the composition is added to be directly infused into humans, and which permit an acceptable post-infusion recoverability of viable RBCs possessing enhanced physiological functioning capabilities and lower rates of clearance from the infused patient's circulation.

#### CITATIONS

D1: BEUTLER E.: "Back to the future in RBC preservation", TRANSFUSION, Vol. 40 (2000), pages 893-895;

D2: BEUTLER E. et al.; "Preservation of red cell 2,3-DPG and viability in bicarbonate-containing medium: The effect of blood-bag permeability", JOURNAL OF LABORATORY AND CLINICAL MEDICINE, Vol. 80, No. 5 (1972)

D3: BENSINGER T. A. et al.: "Prolonged maintenance of 2,3 DPG in liquid blood storage: Use of an internal CO<sub>2</sub> trap to stabilize pH", JOURNAL OF LABORATORY AND CLINICAL MEDICINE, Vol. 89, No. 3 (1977), pages 498-503

D4: WOOD L. A. et al.: "The Effect of Periodic Mixing on the Preservation of 2,3-Diphosphoglycerate (2,3-DPG) Levels in Stored Blood", BLOOD, Vol. 42 No. (1973) pages 17-25;

D5: US 6447987 B1 (HESS J. R. et al.) 10/09/2002

D6: EP 0313808 A2 (AMERICAN RED CROSS [US]) 03/05/1989

D1 is an article that discusses the history of 'accidents' in the blood storage field. The article does not address the role of sodium chloride or any other chloride ion, it simply focuses on ingredients of modern day solutions that were discovered to be important by happenstance. Therefore, we would argue that this reference is not truly relevant to the patentability of the solution of the aqueous composition of the current application.

D2 and D3 are abstracts discussing CO<sub>2</sub> gasses generated by metabolic activity of the stored blood cells. We agree that one of the storage solutions used in D3 is BAGPM, a specific solution that omits chloride ions, and we discuss the particulars of that solution below. Likewise, D4 is an abstract that discusses mixing the bags of cells stored in BAGPM.

BAGPM is a solution containing 101.4 mM of sodium bicarbonate (NaHCO<sub>3</sub>), 14.3 mM sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>), 1 mM bisodium phosphate (Na<sub>2</sub> HPO<sub>4</sub>), 1 mM adenine, 55 mM glucose, and 0.5% mannitol. While BAGPM does not contain sodium chloride or any other ingredient that provides exogenous chloride ions, it does contain sodium carbonate, which is not a necessary component of the aqueous composition of the current application. Further, the concentrations of the ingredients in BAGPM are not the same as the concentrations used in the aqueous composition of the current application. For example, BAGPM uses a very high concentration of sodium bicarbonate, almost 102 mM. In the current application, the broadest range cited is 20 to 130 mM, with a preferred range of 22 to 40 mM, and more specifically, 26 mM. Thus, for all these reasons, BAGPM does not teach the elements of the claimed invention and, therefore, negates the applicability of D2-D4.

In addition, the recited limitation of “wherein the pH buffering system is present in an amount sufficient for the composition to be operable to maintain a pH of a red blood cell (RBC) suspension to which the composition is added at a value sufficient to establish and maintain during a storage period a reaction equilibrium in the red blood cell that favors glycolysis over synthesis of 2,3-diphosphoglycerate (DPG) from 1,3-DPG, thereby generating a net gain in adenosine tri phosphate (ATP) with respect to the reaction equilibrium during the storage period” in the claims is not taught by Wood et al. (D4). For example, Figure 1c of Wood shows that storage in the BAGPM solution does result in an increase in ATP as the storage period progresses. However, the initial decline of ATP (because the BAGPM solution is so alkaline that it forces production of 2,3-DPG in favor of ATP production), is so severe that the ATP levels never fully recover. In other words, at no time does the BAGPM storage solution of Wood ever result in a total net gain of ATP. Therefore, the Wood reference does not teach each and every limitation of the claims of the current application. As such, Applicant argues that the BAGPM solution does not render the claims not novel or lacking inventive step.

D5 describes several blood storage compositions which contain sodium chloride (*see, e.g.,* Table 2). Also, Figure 4 depicts the effect of various concentration of sodium chloride on the health and survival of red blood cells over time. One skilled in the art observing Fig. 4 would not be motivated to eliminate sodium chloride all together. At most, Fig. 4 indicates that high

concentrations of sodium chloride (150 mEq/L) are somewhat detrimental over a long storage period.

Further, D5 teaches using these solutions in large volumes. Such large volumes are impractical to use in the actual storage of red blood cells for blood-banking purposes because, for example, with those solutions, a patient in need of many units of blood would be at a risk of hemodilution. The smaller volumes used in the current application are medically acceptable volumes. The claims of the current application recite the aqueous composition in a volume ratio of 1:4.5 to the volume of collected whole blood, for example, or as a percentage fraction, for example, or as a standard volume used in blood banking, for example. Therefore, the aqueous composition of the current application is an inventive improvement over the compositions described in D5.

D6 teaches a blood storage solution that is, “[e]xcept for the nutrient sugar that is desirably dextrose, citric acid or citric acid derivative, sodium bicarbonate and in certain embodiments, adenine, ... *free of organic compound additives*” (emphasis added) (page 6, lines 8-10 of D6). However, the aqueous composition of the current application contains mannitol, which is, as a sugar, an organic compound. Also, D6 teaches balancing the osmolarity by adjusting the concentrations of electrolytes, such as sodium chloride, potassium chloride, calcium chloride, magnesium sulphate, and monobasic sodium phosphate (page 6, lines 18-23). Thus, D6 fails to teach two important aspects of the aqueous composition of the current application – the use of a membrane-protectant sugar and the specific exclusion of chloride ions.

## **SUMMARY OF THE INVENTION**

Accordingly, the present invention provides novel compositions suitable for the storage and preservation of collected red blood cells. The present inventors surprisingly discovered that substantially eliminating sodium chloride from such compositions, previously considered to be essential to proper operation of storage compositions, provides an increased capacity in the composition for an enhanced pH buffering system which, in turn, provides benefits both in terms of the integrity and physiological functioning quality of the stored and then re-infused red blood cells, and with respect to the length of time the RBCs may be stored with retention of the recoverability and hemolysis levels required under regulatory law for licensing. In addition, the inventive compositions retain their superior performance at conventional volumes, making them particularly suitable for storing red blood cells which may be targeted for infusion into multiply or massively transfused patients.

One embodiment of the present invention provides a composition for storage of red blood cells at 1 to 6° C. The composition consists essentially of: adenine; dextrose; at least one nonmetabolizable membrane-protectant sugar; and a pH buffering system. The pH buffering system comprises sodium bicarbonate and disodium phosphate and is present in an amount sufficient for the composition to have a pH of from 8 to 9. The composition is operable to maintain a pH of a red blood cell (RBC) suspension to which the composition is added at a value sufficient to establish and maintain during a storage period a reaction equilibrium in the red blood cell that favors glycolysis over synthesis of 2,3-diphosphoglycerate (DPG) from 1,3-DPG, thereby generating a net gain in adenosine tri phosphate (ATP) with respect to the reaction equilibrium during the storage period. Another embodiment of the invention provides that the composition is substantially free of sodium chloride.

More specific embodiments of the inventive composition are directed to particular components and amounts thereof, and ranges for the osmolarity and pH of the compositions. Other specific embodiments are directed to inventive compositions that are operable to maintain the pH of the red blood cell within particular value ranges.

A further embodiment of the invention is directed to a suspension of red blood cells comprising the inventive composition.

Method embodiments are also provided. One such embodiment is directed to a method of preserving red blood cells (RBCs) for a storage period. The method comprises: (a) mixing a sample of collected whole blood containing the RBCs to be stored and plasma with an anticoagulant solution, thereby forming a suspension of collected whole blood; (b) treating the suspension of collected whole blood to deplete the plasma and concentrate the RBCs, thereby forming packed RBCs; (c) mixing the packed RBCs with an amount of a composition sufficient to form a suspension of RBCs having 35 % to 70 % RBCs by volume; (d) cooling the suspension of RBCs to 1 to 6° C; and (e) storing the cooled suspension of RBCs according to standard bank procedures. The composition consists essentially of: adenine; dextrose; at least one nonmetabolizable membrane-protectant sugar; and a pH buffering system. The pH buffering system comprises sodium bicarbonate and disodium phosphate and is present in an amount sufficient for the composition to have a pH of from 8 to 9. The composition is operable to maintain a pH of a red blood cell (RBC) suspension to which the composition is added at a value sufficient to establish and maintain during a storage period a reaction equilibrium in the red blood cell that favors glycolysis over synthesis of 2,3-diphosphoglycerate (DPG) from 1,3-DPG, thereby

generating a net gain in adenosine tri phosphate (ATP) with respect to the reaction equilibrium during the storage period. More specific embodiments are also provided.

Additional embodiments are provided which are directed to methods of using the inventive compositions to improve red blood cell (RBC) membrane maintenance and suppress RBC apoptosis during a storage period, to decrease red blood cell (RBC) fragility and suppress RBC hemolysis during a storage period, and to increase viability of red blood cells (RBCs) subsequent to a storage period and after infusion into a patient in need of such an infusion, and decrease a rate of post-infusion clearance of the RBCs by the patient.

Compositions and RBC suspensions produced in accordance with the invention provide a storage period for RBCs throughout which a sufficiently therapeutic amount of the RBCs is recoverable and are directly infusible into patients without further processing in accordance with known standards established and recognized for transfusion of RBCs.

These and additional embodiments and aspects of the present invention will be more fully appreciated by reference to the brief description of the figures, detailed description of the preferred embodiments and examples provided below.

### **BRIEF DESCRIPTION OF THE FIGURES**

**Figure 1:** is a graphical representation of the results of a pooling study showing the effects on RBCs of storage as a function of time in weeks, in 4 different additive solution compositions: 1) AS-3, 110 mL volume (-◇-); EAS-61, 170 mL volume (-◆-); EAS-78, 170 mL, (-●-); and EAS-81, 110mL, (-○-). Bicarbonate-containing compositions, represented by circles, yield higher ATP concentrations, as illustrated in panel A, than the equivalent volume compositions without bicarbonate, represented by the diamonds. These compositions are also associated with higher lactate concentrations (panel D), higher extracellular and intracellular pH (panels E and F), and higher bicarbonate and PCO<sub>2</sub> concentrations (panels E and F). Higher volume compositions, represented by the solid figures, demonstrate reduced hemolysis and reduced storage hematocrit, illustrated in panels B and C, respectively.

**Figure 2:** illustrates the in vivo recovery of red blood cells sampled 24 hours after re-infusion into a subject for RBCs stored in EAS-81 for 6 weeks (n=6), EAS-81 for 8 weeks

(n=6), compared to a historic control, the licensure study for AS-3 published by Simon et al. in 1985. Both studies used the  $^{51}\text{Cr}$  single-label method.

## **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

The invention generally relates to compositions and methods associated with the storage of red blood cells (RBC). In particular, it relates to novel additive solution compositions and related methods for storage of RBCs that have been separated from whole blood collected in citrate phosphate dextrose (CPD) solution, its variant, citrate phosphate double dextrose (CP2D) solution, or by aphaeresis (removal of whole blood from a patient or donor) in acid citrate dextrose (ACD) or similar solutions.

For purposes of this invention, the term "recovery" is used herein to indicate the fraction of stored RBCs that remains in circulation for 24 hours, after re-infusion into the original human donor.

As used herein, "chloride" refers to anionic chloride. Thus, the term "chloride" includes anionic chloride and the salt forms thereof, such as may be formed from chloride anion(s) and physiologically-acceptable cation(s). The term "chloride" is not intended to include compounds wherein the chloride atom is covalently bonded to, for example, a carbon atom in an organic molecule.

As used herein, the phrase "physiologically-acceptable buffering agent" refers to buffering agents which yield cations and anions either normally found in the blood, plasma, or serum of a human, or that may be tolerated when introduced into a human. Suitable cations include protons, ammonium cations and metal cations. Suitable metal cations include, but are not limited to, the cationic forms of sodium, potassium, calcium, and magnesium, where sodium and potassium are preferred, and sodium is more preferred. An ammonium cation, i.e., a compound of the formula  $\text{R}_4\text{N}^+$  where R is hydrogen or an organic group, may be used so long as it is physiologically acceptable. In a preferred embodiment, the cation is selected from hydrogen (i.e., proton), sodium, potassium, calcium, magnesium, and combinations thereof. As used herein, "buffering agent" refers to an agent that adjusts and regulates the pH of a composition.

The inventive compositions disclosed herein are aqueous, that is, they are formulated in water. A preferred water of the invention is treated in order that it is essentially pyrogen-free (i.e., is sterile).

As used herein, "mEq/L" refers to the concentration of a particular component (solute) present in proportion to the amount of water present. More specifically, mEq/L refers to the number of milli-equivalents of solute per liter of water. Milli-equivalents per liter are calculated by multiplying the moles per liter of solute by the number of charged species (groups) per molecule of solute, which is then multiplied by a factor of 1,000.

One embodiment of the present invention provides an aqueous composition for storage of red blood cells at 1 to 6° C. The composition consists essentially of: adenine; dextrose; at least one non-metabolizable membrane-protectant sugar; and a pH buffering system. The pH buffering system comprises a combination of physiologically acceptable buffering agents and must include at least one agent that provides bicarbonate anions, at least one agent that provides phosphate anions, and at least one agent that provides sodium cations. The invention contemplates that a single buffering salt may satisfy more than one of these requirements.

It is well known in the red blood cell preservation arts that the concentration of ATP in the red blood cell suspension system is the best correlate of the health of the system. The red blood cell generates ATP through glycolysis via the glycolytic conversion of d-glucose (dextrose) ultimately to lactate. Hence, the concentration curve of lactate is a good indicator of ATP synthesis as well. Regardless of the preservation capacity of the system, red blood cells have a finite life span and the collected red blood cells include a normal distribution of red blood cell ages and proximities to natural death. As no new RBCs are entering the preservation system, there is a limit to the maximum storage period duration that will provide the requisite post-re-infusion recovery percentage. Hence, the ATP-generating capacity of the system as a whole will decrease over time, though; it is typical to see an initial increase upon addition of an additive fluid as it provides nutrients in higher than natural concentrations and the RBC's initially undergo "swelling," which is associated with decreased ATP utilization as well.

Without being bound by theory, it is believed that when stored in additive solution in accordance with the invention, the increased volume of nutrient solution allows an increased mass of substrate to be delivered at acceptable concentrations while providing solute for dilution of metabolic waste products thereby reducing feedback inhibition of glucose metabolism. It is further postulated that another feature of the additive solutions of the invention is that they produce swelling of the RBCs initially followed by a gradual reduction of red cell volume during storage. Such a process has been called "regulated volume decrease." It is hypothesized that during this process either the tyrosine phosphatase activity present in the RBC is suppressed or the tyrosine

kinase is activated. Both of these enzymes have been demonstrated to be abundant in the membranes of these cells (Zipser, Y. and Kosower, N. S. (1996) *Biochem. J.* 314:881; Mallozzi C. et al. (1997) *FASEB J.* 11:1281). It is anticipated that the net phosphorylation of the band 3 protein in the RBC membrane would result in the release of phosphofructokinase, aldolase and glyceraldehyde-3-phosphate dehydrogenase in the cytoplasm from their bound state to band 3 (Harrison, M. L. et al. (1991) *J. Biol. Chem.* 266:4106; Cossins, A. R. and Gibson J. S. (1997) *J. Exper. Biol.* 200:343; Low, P. S. et al. (1993) *J. Biol. Chem.* 268:14627; Low, P. S. et al. (1995) *Protoplasma* 184:1961. The availability of these three enzymes in the glycolytic pathway would be expected to increase the metabolism of glucose by the RBC, thereby promoting the levels of ATP synthesis and ATP concentration in the RBCs. So, the goal of formulating additive solution compositions is to maintain the ATP synthesis at as high a rate as possible for as long duration as possible.

The present inventors discovered that a key to maximizing the ATP synthesis of the system is to keep the RBC intracellular pH at a level as close to 7.2 as possible without actually reaching it. During storage, the ATP concentration characteristically remains level or even increases for a period of time early in storage and then declines. When the RBC ATP concentration falls below 2  $\mu\text{mol/g Hb}$ , RBC recovery is typically below 75%. RBC's lose 2,3-DPG early in storage. The starting concentration is characteristically 15  $\mu\text{mol/g Hb}$  or 1.1  $\text{mol/mol Hb}$ . The concentration typically falls to one-tenth the starting amount in 7 to 10 days. The rate of synthesis of 2,3-DPG is a function of pH, occurring in excess above pH 7.2 but with breakdown favored below that pH. Attempts to increase 2,3-DPG syntheses by increasing storage-system pH have been limited by the mole for mole loss of ATP synthesis with each 2,3-DPG molecule formed. Thus, raising RBC 2,3-DPG concentrations, something previously considered to be desirable, actually tends to reduce RBC storage time.

A more acidic environment diminishes RBC metabolism. The pH of 7.2 is the point wherein a mechanism, known as the Rappaport shunt (see Hess et al. "Alkaline CPD and the preservation of red blood cell 2,3-DPG" (2002) *Transfusion*, 42:747-752) is triggered whereby 2,3-DPG is synthesized from 1,3-DPG, consuming the phosphate needed for the synthesis of ATP and, additionally, routing around a glycolytic step which produces two of the glycolytically generated ATPs. The net effect to the system is a depletion of ATP. If the intracellular pH can be maintained below 7.2, the shunt is effectively closed down and ATP synthesis is maximized. In a natural state, the shunt operates to some extent and the production and maintenance of some 2,3-DPG is important to other cellular events. However, the present inventors discovered that for

purposes of preservation of the red blood cell during storage outside of the in vivo environment, minimization of the shunt operation is desirable.

Therefore, embodiments of the present inventive composition provide that the pH buffering system is present in an amount sufficient for the composition to be operable to maintain a pH of a red blood cell (RBC) suspension to which the composition is added at a value sufficient to establish and maintain during a storage period a reaction equilibrium in the red blood cell that favors glycolysis over synthesis of 2,3-diphosphoglycerate (DPG) from 1,3-DPG, thereby generating a net gain in adenosine tri phosphate (ATP) synthesis with respect to the reaction equilibrium during the storage period. A specific embodiment of the presently inventive compositions provides that the composition is operable to maintain the pH of the RBC suspension to which the composition has been added at between 6.4 and 7.4. In more specific embodiments, the composition is operable to maintain the pH of the red blood cell (RBC) suspension to which the composition has been added at between 7.0 and less than 7.2. In very specific embodiments the composition is operable to maintain the pH of the red blood cell (RBC) suspension to which the composition has been added at a value greater than 7.1 and less than 7.2.

The present inventors have formulated additive solution compositions substantially free of chloride that surprisingly yields no negative effect on the system and permits the addition of increased amounts of the buffering system to provide additional pH buffering. One embodiment of the invention is directed to an aqueous composition for storage of red blood cells at 1 to 6° C as well. This composition comprises: adenine; dextrose; at least one nonmetabolizable membrane-protectant sugar; and a pH buffering system. The pH buffering system comprises a combination of physiologically acceptable buffering agents including at least one agent providing bicarbonate anions, at least one agent providing phosphate anions, and at least one agent providing sodium cations. The pH buffering system is present in an amount sufficient for the composition to be operable to maintain a pH of a red blood cell (RBC) suspension to which the composition is added at a value sufficient to establish and maintain during a storage period a reaction equilibrium in the red blood cell that favors glycolysis over synthesis of 2,3-diphosphoglycerate (DPG) from 1,3-DPG, thereby generating a net gain in adenosine tri phosphate (ATP) with respect to the reaction equilibrium during the storage period. The composition is substantially free of exogenously derived chloride ions. As used herein, "substantially free of exogenously derived chloride ions" is defined as whatever the concentration of chloride ions is given that no source of chloride ions has been added to the composition.

Additional embodiments are directed to a suspension of red blood cells comprising any of the inventive compositions, and embodiments wherein the suspension is suitable for direct infusion into a patient in need of such an infusion.

Further embodiments of the inventive composition provide that the at least one agent providing sodium cations is selected from the group consisting of sodium bicarbonate, disodium phosphate, and combinations thereof. In a more specific embodiment the at least one agent providing bicarbonate anions is sodium bicarbonate. Additional embodiments provide that the at least one agent providing phosphate ions is selected from the group consisting of sodium phosphate, disodium phosphate, trisodium phosphate, and combinations thereof. In more specific embodiments the at least one agent providing phosphate ions is disodium phosphate. In other embodiments of the inventive composition the combination of physiologically acceptable buffering agents additionally comprises at least one agent providing a physiologically acceptable cation selected from the group consisting of  $H^+$ , potassium, ammonium, magnesium and combinations thereof.

In a further embodiment of the present inventive compositions, the at least one non-metabolizable membrane-protectant sugar is mannitol. Some sugar alcohols, in particular the monosaccharide-derived sugar alcohols (e.g., sorbitol, mannitol, xylitol, erythritol), are small hydrophilic molecules that appear to diffuse readily through some lipid barriers and may play an important role in cellular stability. Mannitol, in particular, is a known antioxidant that acts as a hydroxyl radical scavenger in vivo. It appears to play a substantial role in the maintenance of cell membrane integrity and is considered a membrane-protectant sugar. Other small polyols may also function as membrane protectant sugars. It is significant to note that glucose and mannitol have the same mole weight, that is, 180g/mole. Sugar alcohols are not metabolized by the red blood cell.

As used herein, the reported osmolarity is an empirically derived value. Osmolarity is a measure of the osmotic pressure exerted by a solution across a perfect semi-permeable membrane (one which allows free passage of water and completely prevents movement of solute) compared to pure water. Osmolarity is dependent on the number of particles in solution but independent of the nature of the particles. The osmolarity of a simple solution is equal to the molarity times the number of particles per molecule. Real solutions may be much more complex. Proteins with many equivalents/L may only contribute a small amount to the osmolarity, since they consist of a few very large "particles". Not all ions are free in a solution. Cations may be bound to other

anions or to proteins. Not all the solution volume is aqueous. To be truly accurate, all these factors should be included in the calculation.

Tonicity, a value highly related to osmolarity and somewhat more useful for describing biocellular conditions, is a measure of the osmotic pressure that a substance can exert across a cell membrane, compared to blood plasma. Osmolarity measures the effective gradient for water assuming that all the osmotic solute is completely impermeant. It is simply a count of the number of dissolved particles. A 300 mM solution of glucose and a 150 mM solution of NaCl each have the same osmolarity, for example. However, a cell placed in each of these solutions would behave very differently. Tonicity is a functional term describing the tendency of a solution to resist expansion of the intracellular volume.

Additional embodiments provide that the inventive compositions have an osmolarity of from 200 to 310 mOsm. In more specific embodiments the compositions have an osmolarity of from 221 to 280 mOsm. In a very specific embodiment the osmolarity is 270 mOsm.

As noted, RBCs metabolize glucose (d-glucose = "dextrose") to make ATP. The waste products are lactate and protons. The protons accumulate, driving down the pH and inhibiting further metabolism. Bicarbonate has been suggested as a buffer system wherein it combines with the protons and, in the presence of RBC carbonic anhydrase, is converted to water and carbon dioxide. In a storage container that permits diffusion of the carbon dioxide, the reverse reaction is prevented and the reaction is driven toward the formation of CO<sub>2</sub>. A buffering system based on bicarbonate has considerable capacity. Bicarbonate in physiologic concentrations in the additive solution creates the pCO<sub>2</sub> in the solution that drives the diffusion of up to 1 to 2 mmol of CO<sub>2</sub> from a 600 mL PVC bag each week. However, previous attempts to formulate RBC storage additive solutions with bicarbonate have failed with respect to increasing ATP synthesis and prolonging the effective storage period. For instance, Beutler (BAG-PM) described the addition of bicarbonate to RBC storage solutions, but failed to control for a high pH that led to rapid ATP depletion.

In discovering that saline is not a necessary ingredient to RBC additive solution compositions, and that the concentration of dextrose could be lowered without negative effects on ATP synthesis, the present inventors were able to utilize the resultant increased "play" in solution parameters to increase and fine-tune the pH buffering system. The presently disclosed buffering system provides not only an initially appropriate pH to the additive solution composition, but is able to impart to the RBC suspension a pH that, in turn, modulates the intracellular pH of the RBC

to maximize ATP synthesis. The buffering system achieves these pH modulation targets over the storage period. Hence, the buffering capacity or strength of the pH buffering system is deliberately controlled. One embodiment of the present inventive compositions provide that the composition have a pH of from 8 to 9. In more specific embodiments the pH is from 8.2 to 8.8. In even more specific embodiments the pH of the composition is from 8.4 to 8.6, and in a very specific embodiment the pH of the composition is 8.5. Another embodiment is directed to the inventive compositions wherein the buffering system has a buffering capacity in the red blood cell (RBC) suspension to which the composition is added which increases by at least 2 mEq between a pH of 6.5 and 7.2 over a storage period of 6 weeks. The presently disclosed buffering system should provide a buffering capacity of at least this value, but is capable of providing even greater buffering capacities to the RBC suspension thereby lengthening the storage period even further.

The present inventors determined ranges for the necessary composition ingredients that permit the instantly disclosed advantages. In one embodiment of the inventive composition, the composition comprises adenine in an amount of 1-3 mM, dextrose in an amount of from 20 to 115 mM, un-metabolizable membrane-protectant sugar in an amount of 15 to 60 mM, sodium bicarbonate in an amount from 20 to 130 mM, and disodium phosphate in an amount of from 4 to 20 mM. In a more specific embodiment the composition comprises adenine in an amount of 2 mM, dextrose in an amount of from 60 to 100 mM, unmetabolizable membrane-protectant sugar in an amount of 40 to 60 mM, sodium bicarbonate in an amount of from 22 to 40 mM, and disodium phosphate in an amount of from 7 to 15 mM. In an even more specific embodiment the composition comprises adenine in an amount of 2 mM, dextrose in an amount of 80 mM, unmetabolizable membrane-protectant sugar in an amount of 55 mM, sodium bicarbonate in an amount of 26 mM, and disodium phosphate in an amount of 12 mM, and the composition has a pH of 8.5.

The present invention also provides method embodiments. In one such embodiment a method of preserving red blood cells (RBCs) for a storage period is provided. The method comprises: (a) mixing a sample of collected whole blood containing the RBCs to be stored and plasma with an anticoagulant solution, thereby forming a suspension of collected whole blood; (b) treating the suspension of collected whole blood to deplete the plasma and concentrate the RBCs, thereby forming packed RBCs; (c) mixing the packed RBCs with an amount of an aqueous composition sufficient to form a suspension of RBCs having 35 % to 70 % RBCs by volume; (d) cooling the suspension of RBCs to 1 to 6° C; and (e) storing the cooled suspension of RBCs according to standard bank procedures known in the art. The aqueous composition consists essentially of: adenine; dextrose; at least one non-metabolizable membrane-protectant sugar; and a

pH buffering system. The pH buffering system comprises a combination of physiologically acceptable buffering agents including at least one agent providing bicarbonate anions, at least one agent providing phosphate anions, and at least one agent providing sodium cations, wherein the pH buffering system is present in an amount sufficient for the composition to be operable to maintain a pH of a red blood cell (RBC) suspension to which the composition is added at a value sufficient to establish and maintain during a storage period a reaction equilibrium in the red blood cell that favors glycolysis over synthesis of 2,3-diphosphoglycerate (DPG) from 1,3-DPG, thereby generating a net gain in adenosine tri phosphate (ATP) synthesis with respect to the reaction equilibrium during the storage period. The solution is divided in manufacture to separate the dextrose and the phosphate and bicarbonate during heat sterilization.

RBCs useful in the present invention are those that have been separated from their plasma and resuspended in an anticoagulant solution in the normal course of component manufacture. Briefly stated, a standard whole blood sample (450 +/- 45 ml) containing RBCs and plasma is mixed with an anticoagulation solution (63 ml) to form a suspension of whole blood. Proportional increases or decreases in solution volumes to reflect different donor blood volumes such as 400 +/- 40 ml-500 +/- 50 ml can also be used. The whole blood suspension is thereafter centrifuged to separate the RBCs from the blood plasma thereby forming packed RBCs. The performance of the overall process is improved by leukocyte reduction using conventional techniques.

Suitable anticoagulants include conventional anticoagulants known for storage of RBCs. Preferably; the anticoagulants include citrate anticoagulants having a pH of 5.5 to 8.0, e.g. CPD, half-strength CPD and the like. The most preferred anticoagulant is CPD.

The RBC suspension is then generally stored in standard polyvinyl chloride (PVC) blood storage bags using either the collection bag or PVC transfer packs of different sizes depending on the volume of the stored aliquot. The RBC suspension is stored at 1 to 6° C according to standard blood bank procedure as described in Clinical-Practice of Blood Transfusion editors: Petz & Swisher, Churchill-Livingston publishers, N.Y., 1981. In a specific embodiment of the inventive method, the suspension of RBCs is suitable for direct infusion into a patient in need of such an infusion. While PVC blood storage bags are the industry-approved standard; the present invention contemplates storage in a wide variety of bags adapted for RBC suspension storage, for example, by including appropriate plastisizers as needed. Ingredients related to the bag or container component of RBC storage technology are not discussed herein but it will be readily apparent to one of ordinary skill in the art that many container technologies may be employed to practice the present invention.

The additive solutions of the invention can also be used to rehydrate lyophilized RBC or in the thawing of stored frozen blood or blood component, e.g. RBC.

In specific embodiments of the inventive method of preserving RBCs, the at least one non-metabolizable membrane-protectant sugar is a monosaccharide derived sugar alcohol and in a more specific embodiment the non-metabolizable membrane-protectant sugar is mannitol. In additional embodiments of the method, the at least one agent providing sodium cations is selected from the group consisting of sodium bicarbonate, disodium phosphate, and combinations thereof. In specific embodiments the at least one agent providing bicarbonate anions is sodium bicarbonate. Further embodiments are directed to the inventive method of preserving RBCs wherein the at least one agent providing phosphate ions is selected from the group consisting of sodium phosphate, disodium phosphate, trisodium phosphate, and combinations thereof, and in more specific embodiments that at least one agent providing phosphate ions is disodium phosphate. In other embodiments of the inventive method the combination of physiologically acceptable buffering agents additionally comprises at least one agent providing a physiologically acceptable cation selected from the group consisting of  $H^+$ , potassium, ammonium, magnesium and combinations thereof.

Further embodiments are directed to the inventive method of preserving RBCs wherein the composition has an osmolarity of from 200 to 310 mOsm. In specific embodiments the osmolarity is from 221 to 280 mOsm, and in a very specific embodiment the osmolarity is 270 mOsm. In other embodiments inventive methods are provided wherein the composition has a pH of from 8 to 9. In specific embodiments the pH is from 8.2 to 8.8 and in more specific embodiments the pH of the composition is from 8.4 to 8.6. In a very specific embodiment the pH of the composition is 8.5. An additional embodiment of the inventive method of preserving RBCs provides that the buffering system has a buffering capacity in the red blood cell (RBC) suspension to which the composition is added which increases by 2 mEq between a pH of 6.5 and 7.2 over a storage period of 6 weeks.

The present invention also provides embodiments of the inventive method of preserving RBCs wherein the composition is operable to maintain the pH of the red blood cell (RBC) suspension to which the composition has been added at between 6.4 and 7.4. In specific method embodiments the composition is operable to maintain the pH of the red blood cell (RBC) suspension to which the composition has been added at between 7.0 and less than 7.2, and in even more specific method embodiments the composition is operable to maintain the pH of the red

blood cell (RBC) suspension to which the composition has been added at a value greater than 7.1 and less than 7.2.

Methods according to the present invention directed to specific ranges of the necessary ingredients of the composition are also provided. In one method embodiment the composition comprises adenine in an amount of 1-3 mM, dextrose in an amount of from 20 to 115 mM, unmetabolizable membrane-protectant sugar in an amount of 15 to 60 mM, sodium bicarbonate in an amount from 20 to 130 mM, and disodium phosphate in an amount of from 4 to 20 mM. In a more specific embodiment the composition comprises adenine in an amount of 2 mM, dextrose in an amount of from 60 to 100 mM, unmetabolizable membrane-protectant sugar in an amount of 40 to 60 mM, sodium bicarbonate in an amount of from 22 to 40 mM, and disodium phosphate in an amount of from 7 to 15 mM, and in a very specific embodiment the composition comprises adenine in an amount of 2 mM, dextrose in an amount of 80 mM, unmetabolizable membrane-protectant sugar in an amount of 55 mM, sodium bicarbonate in an amount of 26 mM, and disodium phosphate in an amount of 12 mM, and further wherein the composition has a pH of 8.5.

In accordance with the method of the invention, additive solution is added to the packed RBC suspension in an amount sufficient to provide a therapeutically effective amount of recoverable RBCs in the cell suspension. Preferably, the additive solution is added at a volume ranging from 60 ml to 400 ml, preferably 100 to 150 ml, most preferably 110 ml. The solution is typically used in a 1:4.5 volume ratio of solution to whole blood collected (100mL for a 450mL whole blood collection, 111 mL for a 500 mL whole blood collection, or equivalent). In specific embodiments of the present inventive methods of preserving RBCs, the volume ratio of the composition to the collected whole blood is 1:4.5. In a more specific embodiment the volume of the composition is 110 mL and the volume of the collected whole blood is 500 mL.

The RBC volume fraction in the cell suspension, i.e. after addition of additive solution, is 27 to 70% of the total suspension. More preferably, the RBC volume fraction in the cell suspension is 35 to 50%. Most preferably, the RBC volume fraction in the cell suspension is 43% of the total suspension.

Over the course of the storage period the present inventors monitored and collected data relevant to the health of the red blood cell. As noted in figures 1 and 2, storage according to the present invention resulted in superior red blood cell quality for longer durations as smaller volumes. As noted above, the blood storage "lesion" is an apoptotic event and the red blood cell

membrane undergoes physiological and morphological changes commensurate with programmed cell death. Over the course of the storage period, it is known that the red blood cell membrane surface area decreases so that its shape changes from the biconcave shape that permits maximum surface area per volume, facilitating diffusion of gases and nutrients, to a more spherical shape characteristic of a dying, fragile cell. The red blood cell membrane is initially flexible and deformable, facilitating passage through small capillaries. This overall shape change is accompanied by the pinching off from the membrane of microvesicles, forming spicules on the outer surface of the red blood cell, so that a cell at the end stage this process, upon observation, resembles a spiny urchin (hence the process is referred to as an echinocytic change and the final form prior to lyses is called an echinocyte). The ensuing fragility eventually leads to lyses and death of the cell. Determining hemolysis rate permits an indication of the scope and severity of this activity. In addition to engendering unacceptable levels of hemolysis during storage, these morphological changes trigger clearance mechanisms within a recipient patient's body upon re-infusion of the stored red blood cells, decreasing the post-infusion recovery and decreasing the efficiency of the transfusion. Employment of the compositions according to the present invention, and methods of preserving red blood cells which utilize them, leads to a decreased osmotic fragility, and a decreased rate of hemolysis. This corresponds to an increase in retention of cell membrane surface area and morphological state, and, therefore, an increase in recovery of viable red blood cells and a decrease in post-infusion clearance of the re-infused red blood cells from the recipient patient's body.

One embodiment of the present invention provides a method of improving red blood cell (RBC) membrane maintenance and suppressing RBC apoptosis during a storage period. The method comprises storing the RBCs during the storage period in suspensions to which the inventive compositions have been added. In a specific embodiment, the microvesicular concentration is reduced 75% from concentrations observed in red blood cells stored for the same storage period in RBC suspensions comprising AS-3.

Another embodiment of the present invention provides a method of decreasing red blood cell (RBC) fragility and suppressing RBC hemolysis during a storage period. The method comprises storing the RBCs during the storage period in suspensions to which the inventive compositions have been added. A further embodiment is directed to methods of increasing viability of red blood cells (RBCs) subsequent to a storage period and after infusion into a patient in need of such an infusion, and decreasing a rate of post-infusion clearance of the RBCs by the patient. The methods comprise storing the RBCs during the storage period in suspensions to which the inventive compositions have been added.

The inventive additive solution composition confers several advantages over prior art additive solutions. The red blood cells stored therein may be stored longer, at least 8 weeks with better radioactive chrome-labeled RBC 24 hour in vivo recovery, than any presently licensed solution. Use of the inventive compositions clearly diminishes the scope and severity of the storage lesion. During the storage period, the red blood cells exhibit an acceptable range of hemolysis: 0.2% at 6 weeks and 0.4% at 8 weeks, all below the FDA limit of 1% at the end of licensed storage. Red blood cells stored in the inventive additive solutions exhibit less membrane loss during storage as demonstrated by lower concentrations of membrane microvesicles and less osmotic fragility. Preservation of membrane during storage is expected to help RBC stored in solution to have better flow properties than cells that have lost more membrane. Retention of normal membrane physiology also suppresses the mechanisms present in the recipient-patient's body that trigger selective clearance from circulation and destruction of red blood cells. Hence, the re-infused red blood cells last longer in the recipient-patient and enhanced longer-term recovery of the re-infused red blood cells is possible. Moreover, enhanced longer-term survival of infused red blood cells should lessen the need for repeat transfusions lowering associated risks to the patient. The inventive solutions also permit higher RBC ATP concentrations during the late phases of storage, which is expected to allow better RBC ATP secretion and therefore improved flow and longevity after the cells are transfused. Pragmatically, the present solutions are useful while working with conventional collection systems that collect whole blood in CPD or CP2D for the production of fresh frozen plasma and random donor platelets, or for the collection of fresh whole blood in emergencies.

## **EXAMPLES**

**Table 1**

Compositions of the Tested Additive Solutions

|                                      | <b>AS-3</b> | <b>EAS-61</b> | <b>EAS-76v6</b> | <b>EAS-81</b> |
|--------------------------------------|-------------|---------------|-----------------|---------------|
| <b>NaCL</b>                          | 70          | 26            | 30              |               |
| <b>NaHCO<sub>3</sub></b>             |             |               | 30              | 26            |
| <b>NaH<sub>2</sub>PO<sub>4</sub></b> | 23          |               |                 |               |
| <b>Na<sub>2</sub>HPO<sub>4</sub></b> |             | 12            | 9               | 12            |
| <b>Adenine</b>                       | 2           | 2             | 2               | 2             |
| <b>Na<sub>3</sub>Citrate</b>         | 18          |               |                 |               |

|                 |     |     |     |     |
|-----------------|-----|-----|-----|-----|
| <b>Dextrose</b> | 55  | 110 | 50  | 80  |
| <b>Mannitol</b> |     | 55  | 30  | 55  |
| <b>Volume</b>   | 110 | 170 | 170 | 110 |
| <b>pH</b>       | 5.8 | 8.3 | 8.4 | 8.5 |

### **Example 1**

This example illustrates a performance profile and the advantages of one embodiment of the inventive additive solution composition, designated as EAS-81 (see Table 1). EAS-81 and comparative example AS-3 (Nutricel, Pal Biomedical) are both provided in conventional volumes, while comparative examples EAS-61 and EAS-76v6 are provided in more dilute, larger volumes. EAS-81 and EAS-76v6 both comprise bicarbonate. See Figure 1, wherein the bicarbonate-containing compositions are represented by circles, while those without bicarbonate are represented by diamonds. Higher volume compositions are represented by solid figures while the conventional volume compositions are represented by open figures. All volumes of additive solution compositions disclosed herein are understood to be per 500 mL unit of whole blood for an approximate volumetric ratio of 1:4.5.

The first example is conducted as a pooling study in order to evaluate the effect of storage solution ingredients on RBC metabolism and integrity over the course of 10 weeks of storage in PVC bags. Pooling reduces the largest source of variability in conventional blood storage studies, that is, the innate differences between the RBCs from different donors. Pooling places some of the cells from each donor in every group of the study while maintaining conventional unit size. RBC units, unreactive in the indirect antiglobulin test (IAT) are grouped into sets of 4 ABO-identical units. Each set is then pooled, mixed and aliquoted to make four identical pooled units. One unit from each pool is used in each of the four arms of the study.

The compositions of AS-3 and the EASs are disclosed in Table 1. The EASs are made in the laboratory from USP adenine, sugars and salts available from Sigma Chemicals, St. Louis, MO, and sterilely filtered into one-liter storage bags (Code 4R2032, Baxter Healthcare, Deerfield, IL), as detailed in Hess et al. "The effects of phosphate, pH, and AS volume on RBCs stored in saline-adenine-glucose-mannitol solutions," *Transfusion*, vol. 40: 1000-1006, Aug. 2000. The storage bags are held at 37° C for two weeks. The solutions are then cultured and the cultures incubated for another two weeks. Sterility is confirmed by the absence of bacterial and fungal growth for 7-14 days (SeptiCheck, Becton-Dickinson Microbiology Systems, Sparks, MD), and

the solutions are aliquoted by weight into 600 mL PVC bags (Code 4R2023, Baxter Healthcare Corp., Deerfield, IL). All connections are made using a sterile connecting device (SCD 312, Terumo Medical Corp. Elkton, MD).

#### RBC Unit Preparation:

Standard units of blood ( $500 \pm 55$  mL) are collected in 70 mL of CP2D solution in a triple-bag collection system (Item code #127-23, Pall Corporation, East Hills, NY). Units are leukoreduced with the integral leukoreduction filter. Packed RBCs are prepared by centrifugation for 5 minutes followed by removal of all but 65 mL of the plasma, after which the listed volume of AS or EAS is added sterilely. Units are stored upright at 1-6° C for 10 weeks except for approximately weekly mixing and removal of a 15 mL sample.

#### In Vitro Measurements

Leukoreduction (elimination of white blood cells from the whole blood product) is confirmed by flow cytometry. The total hemoglobin (Hb) concentration is measured with a clinical hematology analyzer (Hematology Cell Counter System Series 9110+, Baker, Allentown, PA). Mean cell volume (MCV) is determined from the RBC count and the microhematocrit of the storage suspension. Supernatant Hb is measured spectrophotometrically using the modified Drabkin assay as discussed in Moore et al., "A micromodification of the Drabkin hemoglobin assay for measuring plasma hemoglobin in the range of 5 to 2000 mg/dl. *Biochem Med* 26:167-173 (1981). Percent hemolysis is determined by measuring the ratio of free to total Hb and the hematocrit.

RBC ATP concentrations are measured in supernatants of deproteinized PRBCs. Cell aliquots are mixed with cold 10% trichloroacetic acid to precipitate blood proteins, centrifuged at  $2700 \times g$  for 10 minutes, and the protein free supernatant frozen at -80° C until tested. ATP is assayed enzymatically using a commercially available test kit (Procedures 366-UV, Sigma Diagnostics, St. Louis, MO).

Blood gases, bicarbonate, and pH are measured or calculated on a blood gas analyzer (Corning 855, Ithaca, NY). Thus, pH is measured at 37° C. Extracellular sodium, potassium, chloride, phosphate, lactate, and glucose are measured on a programmable chemical analyzer (Hitachi 902 Analyzer, Boehringer Mannheim Corporation, Indianapolis, IN). The average degree of RBC shape change from discocytes to echinocytes to spherocytes is measured according

to the method of Usry, Moore, and Manolo, described in "Morphology of stored, rejuvenated human erythrocytes" *Vox Sang* 28:176-183 (1975). Microvesicles are measured in the supernatant at the end of storage. Plasma is untracentrifuged, the vesicle pellet washed three times in saline, and then the total protein is quantified using Bradford's method (BioRad, Richmond, CA).

The results of the comparison testing of the 4 RBC storage solutions are illustrated in figures 1 and 2. In all storage solutions, RBC ATP concentrations increased in the first week of storage (Figure 1A). In the EASs the ATP concentration continued to rise in the second week and remained higher than in the AS-3 for the duration of the study. At eight weeks of storage and beyond, EAS-81 exhibited ATP concentrations equivalent to the increased-volume EAS-61, while EAS-78, having both the increased volume and bicarbonate buffer system, exhibited somewhat higher concentrations.

Hemolysis (the breakdown of red blood cells), increases with duration of storage in all storage solutions (Figure 1b). At all times, however, hemolysis was higher in AS-3. It was modestly reduced in EAS-81 and reduced further in the other EASs. There was no additional reduction of hemolysis with bicarbonate in the high volume solutions. As expected, a lower AS volume resulted in a higher storage hematocrit (Figure 1c) and RBCs lost volume in all of the solutions during storage.

Lactate concentrations were higher across all time points in the bicarbonate-containing EASs (Figure 1d). Total lactate production over 8 weeks of storage was 9 mM in AS-3, 12 mM in EAS-61, 13 mM in EAS-81, and 15 mM in EAS-78. Extra and intracellular pH was also higher at most time points, but the differences in intracellular pH did not achieve statistical significance (Figures 1e and 1f). The time course of bicarbonate loss and PCO<sub>2</sub> rise and fall that provide the buffering in the suspending solution is shown in figures 1g and 1h.

Generally, comparison of the inventive EAS-81 to the AS-3 stored cells reveals greater energy utilization with resultant higher ATP concentrations, less hemolysis better morphology, less microvesiculation, and slightly higher pH in the former. The older EAS's, especially EAS-76v6, perform even better in certain respects, but their greater volume results in greater dilution of the stored RBC which could result in hemodilution of multiply-transfused patient recipients.

## **Example 2**

This example illustrates that an inventive EAS formulation permitted the storage of RBC in conventional-volume additive solution for 8 weeks, with better recovery, lower hemolysis and enhanced membrane preservation over the known 6-week storage solution.

12 volunteer subjects meeting the United States Food and Drug Administration (FDA) and American Association of Blood Banks donor criteria were selected. The subjects donated 500 mL (one unite) of whole blood, which was collected in CP2D primary bag (Item code # 127-23, Pall Corporation, East Hills, NY), and leuko-reduced with all but 65 mL of the plasma removed. EAS-81, 110 mL, was added and the packed RBC solutions were stored upright at 1-6° C with half the samples (n=6) being stored for 6 weeks and half (n=6) for 8 weeks. The week prior to the end of storage, the units were sterilely sampled and cultured. If the culture exhibited no growth, a small aliquot of the stored RBC was labeled with 51-Cr and returned to the donor using the Moroff protocol for single label measurement of RBC recovery.

Graphs of means and standard errors were created and other descriptive statistics of the storage groups were calculated using spreadsheet program software (Excel, Microsoft, Redmond, WA). Lactate production in each of the storage systems was calculated from the beginning and ending concentrations and the storage system volumes were adjusted to account for the hemoglobin and serum protein content. Box plots of RBC recovery values were produced using SyStat Ver. 6 (SPSS Inc., Chicago, IL).

Leuko-reduced RBC stored in CP2D/EAS-81 for 6 weeks had an average autologous 24-hour in vivo recovery of  $85 \pm 5\%$ , with in vivo recovery after 8 weeks at  $87 \pm 2\%$  (Figure 2). The apparent anomaly of higher recovery at longer time is probably a result of the small size of the study and the great variability in the storability of blood from different individuals. These recoveries, however, are superior to any currently licensed storage solution and would meet the criteria for licensure in the US and Europe (that is, exhibiting a recovery greater than 75% and hemolysis less than 1% for the U.S., or a recovery greater than 75% and hemolysis less than 0.8% for Europe). RBC Hemolysis fraction during this study was  $0.2 \pm 0.2$  at six weeks and  $0.4 \pm 0.2$  at 8 weeks. RBC microvesicle protein concentrations were  $8 \pm 4$  at 6 weeks and  $12 \pm 6$  mg/dL RBC at 8 weeks, and thus accounted for only 5% of the RBC hemoglobin loss.

The superior storage performance of the inventive EAS-81 in terms of both duration of storage and improved physiological functioning of the infused product, appears to derive from several bases. First, the novel pH modulating system permits sustained buffering of the pH of the suspended solution across the tested storage period which is sufficient to maintain a pH in the

intracellular space sufficient to drive the internal cell equilibrium toward glycolysis and away from the ATP-consuming production of 2,3-DPG. In addition, the higher extracellular phosphate concentrations in this formulation contribute toward limiting extracellular  $\text{Ca}^{++}$  concentrations, which in turn suppresses several apoptotic processes such as phospholipids scrambling and membrane deformation and loss. This leads to improved post-infusion recovery and a higher membrane quality in the infused RBCs, further diminishing the triggering of rapid in vivo clearance mechanisms in infused patients and resulting a higher percentage of recoverable infused RBCs for an extended period beyond infusion. The EAS-81 formulation, which eliminates NaCl in favor of increasing the amount of the particular buffering compounds while maintaining suitable osmolarity, permits more effective, longer lasting pH modulation of the RBC-additive solution suspension, and more phosphate available to minimize adverse cellular events associated with increasing  $\text{Ca}^{++}$  concentrations and decreasing ATP concentration. Moreover, the ability to formulate EAS-81 in conventional volumes makes it particularly suitable for use in multiply and massively transfused patients.

**What is claimed is:**

1. A method of preserving red blood cells (RBCs) for a storage period comprising:
  - (a) mixing a sample of collected whole blood containing the RBCs to be stored and plasma with an anticoagulant solution, thereby forming a suspension of collected whole blood;
  - (b) treating the suspension of collected whole blood to deplete the plasma and concentrate the RBCs, thereby forming packed RBCs;
  - (c) mixing the packed RBCs with an amount of an aqueous composition sufficient to form a suspension of RBCs having 35% to 70% RBCs by volume;
  - (d) cooling the suspension of RBCs to 1 to 6°C; and
  - (e) storing the cooled suspension of RBCs according to standard bank procedures,

wherein the aqueous composition consists essentially of :

adenine;

dextrose;

at least one non-metabolizable membrane-protectant sugar; and  
a pH buffering system,

wherein the aqueous composition is substantially free of exogenously derived chloride ions, wherein the pH buffering system consists of a combination of physiologically acceptable buffering agents including at least one agent providing bicarbonate anions, at least one agent providing phosphate anions, and at least one agent providing sodium cations, wherein the pH buffering system is present in an amount sufficient for the composition to be operable to maintain a pH of a red blood cell (RBC) suspension to which the composition is added at a value sufficient to establish and maintain during a storage period a reaction equilibrium in the red blood cell that favors glycolysis over synthesis of 2,3-diphosphoglycerate (DPG) from 1,3-DPG, thereby generating a net gain in adenosine tri phosphate (ATP) synthesis with respect to the reaction equilibrium during the storage period, wherein the at least one agent providing sodium cations is selected from the group consisting of sodium bicarbonate, disodium phosphate, and combinations thereof,

wherein the composition comprises adenine in an amount of 1-3 mM, dextrose in an amount of from 20 to 115 mM, unmetabolizable membrane-protectant sugar in an amount of 15 to 60 mM, sodium bicarbonate in an amount from 20 to 130 mM, and disodium phosphate in an amount of from 4 to 20 mM,

wherein the composition comprises adenine in an amount of 2 mM, dextrose in an amount of from 60 to 100 mM, unmetabolizable membrane-protectant sugar in an amount of 40 to 60 mM, sodium bicarbonate in an amount of from 22 to 40 mM, and disodium phosphate in an amount of from 7 to 15 mM.

2. The method of preserving RBCs as recited in claim 1, wherein the composition comprises adenine in an amount of 2 mM, dextrose in an amount of 80 mM, unmetabolizable membrane-protectant sugar in an amount of 55 mM, sodium bicarbonate in an amount of 26 mM, and disodium phosphate in an amount of 12 mM, and further wherein the composition has a pH of 8.5.
3. The method of preserving RBCs as recited in claim 1, wherein the volume ratio of the composition to the collected whole blood is 1:4.5.
4. The method of preserving RBCs as recited in claim 1, wherein the volume of the composition is 110 mL and the volume of the collected whole blood is 500mL.