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Improving Oxygen Binding of Desiccated Human Red Blood Cells

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Abstract

Desiccating human red blood cells (RBCs) to increase their storage life has been the subject of intense research for a number of years. However, drying RBCs invariably compromises their integrity and has detrimental effects on hemoglobin function due to autoxidation. We have previously demonstrated an RBC desiccation and rehydration process that preserves RBC antigenic epitopes better than frozen RBCs. This study expands on those observations by examining what effects this desiccation process has on RBC hemoglobin function with respect to oxygen binding properties. In this paper, we examined RBCs from normal donors which were desiccated to 25% moisture content and stored dry for 2 weeks at room temperature prior to rehydration with plasma followed by structural and functional studies. Our data showed that approximately 98% of the RBCs were intact upon rehydration based on hemolysis assays. Oxygen dissociation curves for the desiccated/rehydrated RBCs showed a left shift compared to fresh RBCs (pO_2 = 17 mmHg vs. 26 mmHg, respectively). The desiccated/rehydrated RBCs also showed an increase in methemoglobin compared to fresh RBCs (4.5% vs 0.9%, respectively). 2,3-Diphosphoglycerate concentration of the desiccated/rehydrated RBCs was reduced by 20%. In conclusion, although this RBC dehydration process preserves RBC integrity and hemoglobin oxygen binding properties better than most other dehydration techniques described so far, further optimization and long-term studies are needed to make this procedure acceptable for human transfusion.

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Keywords

Desiccation, Red Blood Cells, Oxygen Binding, Hemolysis, Methemoglobin, 2,3-Diphosphoglycerate

1. Introduction

Stabilization of RBCs that allows for dried storage at room temperature, without causing prohibitive hemolysis and hemoglobin oxidation, could potentially change the future of blood banking because it offers numerous advantages over 4˚C and frozen storage. Dry storage that could extend the product's shelf life, will ease logistical problems, facilitate efficient transportation and save energy by eliminating the need for refrigeration [1].

Currently, the only viable alternative to storing RBCs at 4˚C is freezing. However, freezing cells in the absence of cryoprotective agents cause irreparable cellular damage because ice crystals puncture membranes and organelles [2]. Apart from the physical damage, ice crystals inflict on cellular structures; the freeze-drying process itself can damage and/or oxidize proteins, including hemoglobin (Hb) [1]. Hb inside RBCs is also vulnerable to oxidation in the freeze-drying process. Oxidized Hb (met-hemoglobin) does not bind oxygen [3]. The oxidation of Hb within the RBCs can also lead to formation of intracellular reactive oxygen species that can damage lipids and structural proteins [4]. Attempts at freeze-drying RBCs have mostly resulted in poor cellular recovery [1] [2] [4].

Many factors can influence recovery of functional RBCs after freeze-drying. This includes addition of cryoprotectants (e.g. glycerol and DMSO), lyoprotectants (e.g. trehalose and polymers), manipulating the operating condition during the drying process (primary and secondary drying) and the rehydration process. As of yet, recovery of intact RBCs after drying and rehydration has been reported to be between 50% and 85% [5]-[8], but because of elevated Hb oxidation, no RBC desiccation procedures meet the standards for transfusion at present [1] [4].

Here, we present a storage process for RBCs that utilizes desiccation. The desiccation process does not involve freezing; thus ice-crystal damage and hemolysis is avoided. The overall desiccation/rehydration process involves several steps including incubation in HemSol™ which stabilizes the RBC morphology prior to desiccation. HemSol™ contains a proprietary mixture of non-toxic high and low molecular weight carbohydrates, including trehalose, mannose and dextrans that protect RBC integrity and protein function during the desiccation and rehydration process. Previously we demonstrated that RBCs desiccated and stored in HemSol™ for over 3 weeks at room temperature can be used, in lieu of fresh RBCs, as standards for hemagglutination assays [8].

This RBC desiccation process did not involve freezing. HemSol™ treated RBCs were dried in a vacuum oven at low temperature (42˚C) until approximately 25% of the water was remaining. That study showed that our HemSol™ desiccation process preserves RBC morphology and blood typing epitopes better than RBCs that had been frozen. This opens up the possibility of using desiccated RBCs to archive serological phenotypes for use in reference laboratories.

In this study we expand on these previous results by examining whether HemSol™ treatment alters the main physiological property of RBCs, namely the oxygen carrying capacity of Hb. RBCs used in this study were stored desiccated in HemSol™ at room temperature for 2 weeks prior to being rehydrated. Our results demonstrate that the desiccated/rehydrated cells have functional Hb. Compared to fresh RBCs, desiccated and rehydrated RBCs were 98% ± 1% intact based on hemolysis and could be used for these assays directly without a washing step. The rehydrated RBCs demonstrated a left shift in the pO_2 (p50) oxygen saturation value compared to fresh control RBCs (17.10 mmHg vs. 26.48 mmHg, respectively). The reasons for this left shift could be due to increased methemoglobin levels (4.5%) and/or decreased 2,3-diphosphoglycerate (DPG) levels (20%).

To our knowledge, this is the first time these types of experiments have been performed on Hb function in RBC that have been desiccated for 2 weeks at RT. This is a promising start for developing a procedure for producing desiccated RBCs suitable for human transfusion, but further studies are needed to optimize this process.

2. Materials and Methods

Red Blood Cells and Morphology: In-dated human blood, no older than 1 - 3 day was obtained from the Red Cross (American Red Cross, Rockville, MD) as packed red cells. Cells were checked for count per unit volume,

morphology, hemolysis and crenated cells. Evaluations were performed according to standard manual methods for counting (hemocytometer), microscopy and digital photo-microscopy. For morphology experiments, the RBCs were divided into 2 equal parts, one part to be used for desiccation process and room temperature storage and the other part was kept refrigerated at 4˚C as a control.

Aliquots of the reconstituted RBCs were diluted 1:10,000 in saline and examined for morphology under a compound microscope at 400× magnification. For each sample, 5 independent fields each containing at least 100 RBCs were examined.

Desiccation and Rehydration of Red Blood Cells: RBCs were mixed with HeMemics' desiccation buffer called HemSol™ as described previously [8]. Briefly, a HEPES buffer pH 7.4 contained a proprietary ratio of low molecular weight sugars (Trehalose, Mannitol and Glucose) and high molecular weight sugar Dextran. For these experiments, RBCs were washed with saline 3 times using low speed centrifugation (100 ×g for 10 minutes) to remove residual plasma. The packed RBCs were then mixed with HemSol™ at 1:1 ration and allowed to equilibrate at room temperature for 30 minutes. Then, 0.5 mL volumes of the red blood cells mixed with HemSol™ were dried in 20 mL screw top glass vials. Specifically, aliquots of the blood-desiccation buffer mix which were dispensed into glass vials were loaded into a vacuum drying oven and dehydrated under mild heat (42˚C) until visibly dry. The percent moisture of the resulting RBC preparations was determined by *gravimetric* drying *method* using the Sartorius MA45 Gravimetric Moisture Balance (PWB, Bradford, MA). The samples were then capped and stored at room temperature (23˚C - 25˚C) until use.

For the rehydration process, the desiccated cells were rehydrated in human plasma. Plasma (1 mL) was added to the cells in the vial and allowed to incubate for 30 minutes at 37˚C with gentle swirling or vial rotation to insure uniform rehydration. To ensure sufficient volume for testing once rehydration was completed, contents of several vials were combined and adjusted to 45% hematocrit with human plasma for testing.

Post-Rehydration Evaluation for Hb-Oxygen Dissociation Curve Analysis: After 2 weeks of storage in HemSol™ at RT, the desiccated blood was reconstituted as described above and oxygen dissociation curve testing was conducted. The Hemox-Analyzer (TSC Scientific Corporation, New Hope, PA) was used to determine the oxygen dissociation curve (ODC) by exposing 50 µL of blood to an increasing partial pressure of oxygen and deoxygenating it with nitrogen gas. A Clark oxygen electrode was used to detect the change in oxygen tension, which was recorded with on the x-axis of an x-y recorder. The resulting increase in oxyhemoglobin fraction was monitored simultaneously by dual wavelength spectrophotometry at 560 nm and 576 nm and displayed on the y-axis. For the assays, reconstituted RBC (45% HCT) prepared in plasma with heparin anticoagulant was kept on wet ice until the assay. Fifty µL of RBC was diluted in 5 µL of Hemox buffer (7.4 ± 0.01). The sample-buffer was drawn into a cuvette and the temperature of the mixture was equilibrated and brought to 37˚C; the sample was oxygenated to 100% with air. After adjustment of the pO_2 value, the sample was deoxygenated with nitrogen; during the deoxygenation process the curve was recorded on graph paper. The pO_2 (p50) value was extrapolated on the x-axis as the point at which O_2 saturation was 50%. Two different lots of desiccated RBCs were tested and from each lot, samples were tested in triplicate.

Determination of Hemolysis: A sample of control and reconstituted RBCs were centrifuged at 3000 ×g for 10 min to pellet the cells. Absorbance of the supernatant was measured at 450 nm and compared to the absorbance of the lysed pellet to give % hemolysis.

Met-Hb Determination: To lyse RBC, 0.5 mL of desiccated/reconstituted RBC at 45% hematocrit was washed in saline 3 times via centrifugation at 500 g for 15 minutes. To the RBC pellet, 5.5 mL of distilled water was added and allowed to stand for 5 min at RT. The samples was diluted with 4 mL of 0.1 M PBS, pH 6.8, mixed well and centrifuged at 3000 ×g for 10 min. The supernatant was removed for Met Hb analysis. Briefly, 0.5 mL of 4% (w/vol) $K_3Fe(CN)_6$ was added to 3 mL RBC supernatant and allowed to sit for 10 min, and A630 nm measured using a spectrophotometer. To calculate baseline, 0.5 mL water was added to a fresh 3 mL sample of RBC supernatant and absorbance was measured at 630 nm. The reaction was then neutralized by the addition of 0.050 mL 5% (w/vol) KCN and after 5 min. absorbance 630 nm was measured again. Met-Hb was calculated as the ratio of the absorbance change at 630 nm after adding KCN to the RBC supernatant. Control RBC sample was treated in the same manner to extract Met-Hb for measurements. All samples were tested in triplicate.

Measurement of 2,3-DPG (DPG): The assay was performed on 2 week old RBC samples stored desiccated in HemSol™ and fresh RBCs using a commercially available human DPG ELISA Kit (antibodiesonline.com, Atlanta, GA). The microtiter plate provided in this kit had been pre-coated with an antibody specific to DPG. Procedures were well described in the protocol provided with the kit. Briefly, the RBC supernatant prepared as de-

scribed in the Met-Hb determination section was added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for DPG. Avidin conjugated to Horseradish Peroxidase (HRP) was added to each well and incubated, followed by addition of TMB substrate solution. The peroxidase reaction was terminated by the addition of a sulphuric acid solution, and A450 was measured in a spectrophotometer. The concentration of DPG in the samples was calculated from a DPG standard curve. Control RBC sample was treated in the same manner to extract Met-Hb for measurements. All samples were tested in triplicate.

3. Results

Red blood cells were desiccated to different levels of dryness and then the percentage of overall hemolysis was determined. Samples were reconstituted with plasma and assayed for hemolysis as described. **Table 1** shows that removal of more than 25% of residual moisture resulted in an increase in cell fragility upon reconstitution with increased time of storage. Thus, based on these observations, the subsequent data were performed on RBC at 25% residual moisture at 2 week storage cut off.

Changes in RBC morphology after the desiccation/rehydration process were evaluated by gross microscopic examination. Typical, healthy red blood cells are round, smooth bi-concave disks with a pink or red color. Other forms of RBCs, such as crenated (partially shrunken) and spherocytes can be present [9]. Crenated RBCs are associated with storage lesion, but also occur in response to high osmotic conditions or drying. Crenation is often reversible and while exposure to the high osmotic load of HemSol™ does cause some crenation, with proper rehydration, most of the crenated cells revert to smooth bi-concave disks after 30 min in human plasma (**Figure 1(a)**). **Figure 1(b)** shows control RBCs kept for 2 weeks at 4˚C.

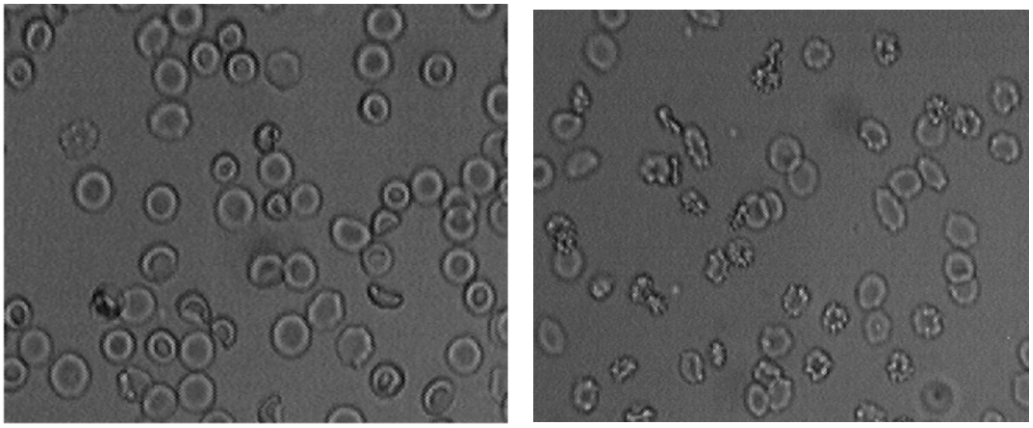
The critical physiologic parameter for RBC preparations is their ability to properly bind, carry and release oxygen. The determinations of this ability are conducted by exposing the RBCs to oxygen to assure complete loading, and then exposure to complete nitrogen to determine the continuous unloading of the oxygen (oxyhemoglobin dissociation curve). Typical oxyhemoglobin dissociation curve determinations are presented in **Figure 2** and demonstrate a sigmoidal curve for both the fresh control RBCs and the desiccated/rehydrated RBCs, indicating cooperative oxygen binding.

Met-Hb is a measure of hemoglobin oxidation and is expressed as a proportion of total hemoglobin. Met-Hb is a naturally occurring oxidized metabolite of hemoglobin; under normal physiologic conditions, or in fresh RBCs, around 1% of total Hb is Met-Hb [10]. In desiccated/reconstituted RBCs, Met-Hb levels were 4.56% ± 0.15% upon reconstitution. Fresh control RBCs showed 0.9% ± 0.12% Met-Hb (**Figure 3(a)**).

Table 1. Correlation of RBC hemolysis to moisture content.

% Residual Moisture	% Hemolysis at Day 1	% Hemolysis at Day 7	% Hemolysis at Day 14	% Hemolysis at Day 21
10 ± 0.8	9.5 ± 1.1	11.4 ± 1.4	11.8 ± 2.2	15.5 ± 2.4
15 ± 0.7	5.5 ± 1.3	6.1 ± 1.1	7.3 ± 1.8	10.1 ± 2.1
20 ± 0.8	2.0 ± 0.7	2.5 ± 0.6	3.0 ± 0.8	5.5 ± 1.2
25 ± 1.0	0.4 ± 0.2	0.5 ± 0.1	0.6 ± 0.3	1.5 ± 0.5
30 ± 1.2	0.3 ± 0.2	0.4 ± 0.1	0.7 ± 0.2	1.8 ± 0.5
35 ± 1.3	0.5 ± 0.1	0.4 ± 0.3	0.4 ± 0.2	0.9 ± 0.3
40 ± 1.3	0.3 ± 0.2	0.5 ± 0.4	0.7 ± 0.3	0.8 ± 0.2
45 ± 1.2	0.5 ± 0.3	0.6 ± 0.2	0.5 ± 0.3	1.0 ± 0.2
50 ± 1.1	0.2 ± 0.1	0.5 ± 0.2	0.7 ± 0.3	0.8 ± 0.2
55 ± 1.3	0.5 ± 0.3	0.4 ± 0.1	0.6 ± 0.2	0.8 ± 0.4
Control RBC	0.4 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.6 ± 0.1

Data from 5 lots of RBC purchased commercially. Each samples were tested in triplicate ± Standard deviation. Samples were pooled from storage and tested at time intervals indicated. Control RBC samples were stored refrigerated in accordance with FDA requirements.



(a) (b)

Figure 1. Light field images of RBCs. (a) RBCs stored desiccated for 2 weeks and reconstituted in human plasma and (b) RBCs stored for 2 weeks at 4˚C (400× magnification).

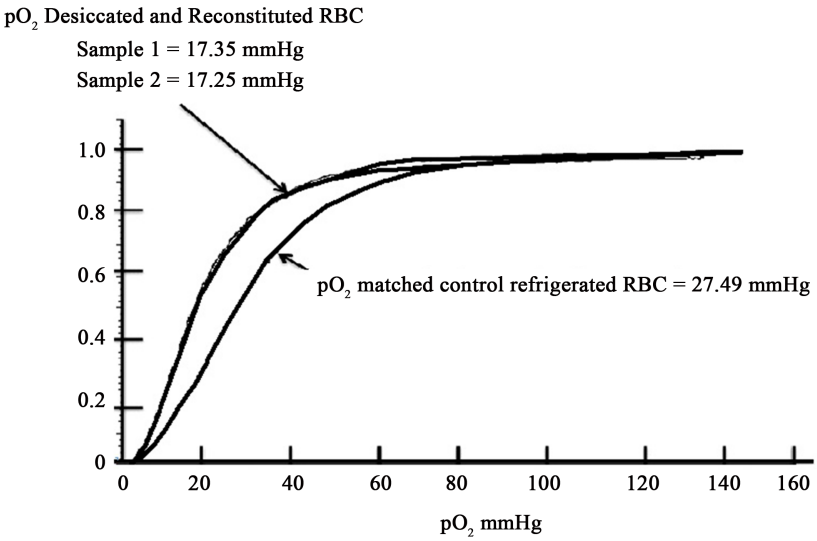
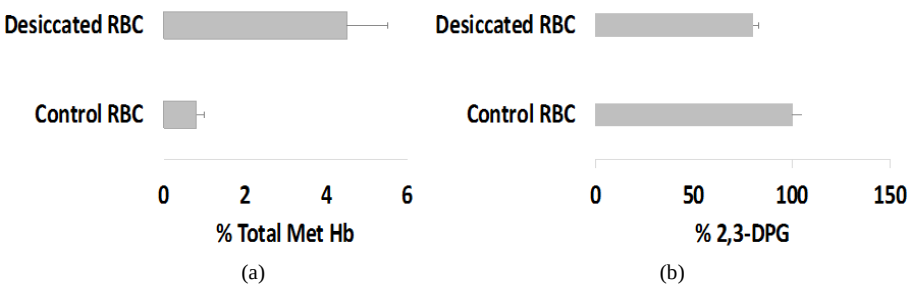


Figure 2. Oxygen binding and release profile for reconstituted RBCs. Dried RBCs were prepared in two separate sets from two different donors and kept for 2 weeks at room temperature before testing. The fresh and rehydrated RBCs (from both sets) showed similar oxygen saturation kinetics (99.4%). The P50 of the rehydrated RBCs (17.35 for set 1 and 17.25 for set 2) were somewhat reduced compared to the fresh RBC (P50 = 27.49). These assays were performed in triplicate.



(a) (b)

Figure 3. Measurements of Met Hb and DPG levels in RBC. (a) Met-Hb content increased in desiccated RBC compared to fresh RBC; (b) The assay for DPG content showed a decrease of approximately 20% for desiccated RBCs upon reconstitution compared to fresh RBCs. These assays were performed in triplicate.

2,3-Diphosphoglycerate (DPG) is an important regulator of Hb oxygen affinity and we measured DPG concentrations in desiccated/rehydrated RBCs stored for 2 weeks at room temperature in HemSol™. Using fresh RBCs as control, we found that the concentrations of DPG in the desiccated/rehydrated RBCs were reduced by 20 % (**Figure 3(b)**).

4. Discussion

RBCs destined for transfusion must be stored refrigerated at 4˚C with a maximum allowable storage time of 42 days [9] [11]. Freezing RBCs and storing them in frozen state is an alternative to cold storage. Subzero temperatures decrease most biochemical reactions and frozen RBCs have been shown to have a longer shelf life [12]. However, the cryopreservation technique has disadvantages because of the need to introduce sometimes toxic antifreezing agents (e.g. DMSO, high concentrations of glycerol) into the blood before the freezing process and removing them before transfusion, all the while maintaining sterility. Because of the labor-intensive and technically demanding nature of processing coupled with the high cost, frozen RBCs are not in routine clinical use. Furthermore, freezing RBCs, even in the presence of cryoprotective agents does not eliminate hemolysis or hemoglobin oxidation. The damage caused by freezing and thawing also leaves behind cellular debris and materials unacceptable for transfusion [1] [13].

Drying RBCs in the absence of freezing step and keeping them functional is even more challenging than freeze-drying [13]. Most biochemistry occurs in an aqueous environment but a few organisms can survive extensive dehydration, a process termed anhydrobiosis. Drying cells without destroying them requires the presence of lyoprotectants. The most common lyoprotectant found in nature is the disaccharide trehalose, although trehalose alone is not sufficient to ensure full integrity upon rehydration [14].

The HemSol™ formulation used in this study contains trehalose along with other carbohydrates that have been shown to be non-toxic in humans. This RBC drying process does not involve freezing and has been shown to preserve RBC integrity and antigenicity better than freezing [8]. For this study we used an improved HemSol™ formulation that recovered 98% ± 1% of the RBCs after reconstitution compared to 50% as we reported previously [8]. In contrast, most studies on drying and reconstituting RBCs have reported recoveries between 20% - 50% [5]-[8].

Before assessing the oxygen binding properties of RBC, we performed experiments to determine the optimal moisture content and storage time for the desiccated RBC. Our data showed that the 25% residual moisture was the optimal level of dryness for RBC using our process. This data also suggested that removal of more than 75% of cell water content may compromise cell membrane structure that could cause higher breakage upon reconstitution. Furthermore, we also showed that beyond 2 weeks of storage, the cells also became more fragile upon reconstitution as shown in **Table 1**. It is possible that a longer storage time at room temperature may further decrease the moisture content of the cells. As such, in this study, we confined our functional studies to 2 weeks in storage of cells at 25% residual moisture.

We examined the morphology of the reconstituted RBCs after a 2 week storage in HemSol™ at room temperature. The RBCs were then hydrated with plasma as described in the materials and methods and imaged under a microscope. **Figure 1(a)** show representative fields of the rehydrated RBCs stored in HemSol™ at room temperature for 2 weeks and control RBCs (**Figure 1(b)**) stored in Adsol™ at 4˚C for 2 weeks, respectively. As seen in this figure, the majority of HemSol™ stored RBCs show typical smooth biconcave structures, while the majority of Adsol stored RBCs showed some crenation. These results demonstrate that the RBCs treated with HemSol™ show less ultrastructural damages than RBCs stored at 4˚C in Adsol™ for the same time period.

The main function of RBCs is to carry oxygen to tissues. The ability of RBCs to bind and release oxygen is influenced by many factors including Hb oxidative state and regulators of Hb oxygen binding [9]. The standard way to measure this property is to perform oxygen saturation studies. **Figure 2** shows oxygen dissociation curves of RBCs stored desiccated in HemSol™ for 2 weeks. The reconstituted RBCs were compared to fresh RBCs and there is a left shift in the HemSol™ treated RBCs compared to fresh RBCs (pO_2 of 26.48 mmHg vs 17.10 mmHg respectively). Both RBC preparations demonstrate a sigmoidal curve, indicating cooperative oxygen binding of hemoglobin. Additionally, both RBC preparations have similar O_2 saturation concentrations, which indicates that most of the Hb in the desiccated/reconstituted RBCs is functional. Interestingly, the pO_2 value of 17 mmHg is comparable to RBCs stored in acid-citrate-dextrose-adenine for approximately 5 days at 4˚C [15].

To further examine Hb oxidation levels in desiccated cells, we measured the levels of methemoglobin (Met-Hb), which is an indication of oxidative stress associated with freeze-drying and desiccation [1] [13]. The heme group in Met-Hb has been oxidized from the oxygen carrying ferrous (Fe^{2+}) state to ferric (Fe^{3+}). Autoxidation of Hb to Met-Hb occurs naturally under normal physiologic conditions, accounting for around 1% of total Hb [3], but prolonged exposure of stored blood to atmospheric oxygen levels can increase Met-Hb levels. Desiccation of RBCs has been shown to increase the Met-Hb levels to 15% - 50% [1] [13]. **Figure 3(b)** shows that the Met-Hb was 4.5% in desiccated/reconstituted RBCs, compared to 0.9% in control RBCs. This data supports the results of **Figure 2**, which indicates that the majority of Hb in the desiccated/resuspended RBCs is functional. Although 4.5% Met-Hb is lower than most desiccation procedures have reported, it is still higher than physiological concentrations (0% - 3%) [3].

Since the data shown in **Figure 2** and **Figure 3(a)** suggest that most of the Hb in desiccated/resuspended is capable of binding oxygen, we examined DPG levels in the HemSol™ treated RBCs to account for the left shift in the oxygen saturation experiment (**Figure 2**). This left shift is consistent with lower concentrations of 2,3-diphosphoglycerate [16].

DPG is an important regulator of Hb oxygen binding. It binds deoxygenated Hb, enhancing release of remaining bound oxygen. We measured the concentrations of DPG in the desiccated/reconstituted RBCs and those results shown in **Figure 3(b)** demonstrate there is a 20% reduction in DPG concentrations in the HemSol™ treated cells. This is consistent with studies of RBC storage lesions, in which the steady depletion of DPG leads to a left shift in Hb oxygen binding properties.

5. Conclusion

Here, we describe a storage process for red cells that utilizes desiccation rather than refrigeration or freezing. The drying process does not involve freezing, thus ice-crystal damage and subsequent debris formation. It is clear that more work is needed to show HemSol™ RBC desiccation as an alternative to regular RBC storage; however, to our knowledge, the present study shows that this process is superior to most other RBC desiccation procedures with respect to preserving Hb oxygen binding properties during desiccation and storage at room temperature for 2 weeks.

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