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Impact of lyophilization on porcine hemoglobin properties.

Mustafa Almosawi
University of Louisville

Impact of Lyophilization on Porcine Hemoglobin Properties

By

Mustafa Almosawi

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Abstract

Blood transfusion is the single most often performed lifesaving procedure in hospitals worldwide. Unfortunately, packed red blood cells (RBCs) used for transfusion can only be stored for 42 days at 4 °C before being discarded due to irreversible damage that occurs during storage. Any reduction in available RBCs for an extended period can lead to blood shortages. To increase the shelf-life of RBCs, we investigated freeze-drying (lyophilizing) in the presence of the non-toxic sugar trehalose as a method for long-term preservation. However, the oxidative stress of the lyophilization and storage processes can compromise the functionality of these cells, and the oxygen-transport protein hemoglobin may convert via oxidation into the nonfunctional methemoglobin (MetHb) form. Therefore, it is necessary to quantify the percentage of MetHb after rehydration to determine the functionality of lyophilized RBCs. Likewise, investigating the allosteric regulation of oxygen binding by hemoglobin is required to establish the suitability of the rehydrated product for transfusion. We found that prevention of oxidation of hemoglobin to methemoglobin during freeze-drying is challenging, and methemoglobin concentration increased during storage of the desiccated RBCs. This increase occurred during storage at high and low humidity and under vacuum. However, RBCs fortified with ascorbic acid before lyophilization and stored at 0% relative humidity showed the lowest methemoglobin content. Moreover, our data demonstrate that allosteric regulation of hemoglobin is maintained after lyophilization and rehydration. Optimizations of the freeze-drying and storage processes are being performed to develop this technology further.

Lay Summary

When you go to the grocery store there are two types of milk that you can buy, bottled liquid milk and powdered dry milk. Bottled milk is in the fridge, must be kept at low temperature and it last no longer than few months before it expires. Dry milk on the other hand, is on the shelf, it can be stored at room temperature, and it last for years – all you need is to rehydrate it with water. Currently blood is more like liquid milk, it must be stored at 4 ^{o}C and can only last for 42 days before it should be discarded. Therefore, complex infrastructure and continuous blood supply must be maintained to avoid blood shortages. This is extremely critical since blood transfusions are the most performed lifesaving procedure – the only thing that can save a patient who has lost blood is blood. Drying blood to a powder form is a potential way to solve this issue, since it will eliminate the need for refrigeration and extend the shelf life of the blood thus buffering against any distribution in the blood supply.

The purpose of our research is to develop blood powder and to make sure that this form of blood will function just as well as liquid blood after rehydration and that it is suitable for transfusion. The research focuses on the function of hemoglobin – the protein responsible for binding oxygen and transporting it throughout the body. The research's goal is to ensure that the hemoglobin of powder blood can bind oxygen in the same way as liquid blood.

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Introduction

Blood transfusions are the single most often used lifesaving procedure in hospitals worldwide [1]. If a patient cannot receive blood during surgery or trauma, death could be the outcome. Unfortunately, red blood cell (RBC) units used for transfusion can only be stored for 42 days at 4 °C before they must be discarded due to irreversible biochemical changes [2]. Therefore, current long-term storage is not practical in emergency situation. Moreover, the temperature sensitivity of pRBCs requires additional costs for maintaining the logistics necessary for refrigerating the blood units.

The complex regulations at the core of the blood distribution system can cause disruptions to the blood supply. For example, the restrictions imposed at the start of the COVID-19 pandemic, where blood donation had dropped to unsustainable levels nationwide, have led to blood shortages [3]. Furthermore, some rural and developing regions worldwide lack the cooling chain logistics and infrastructure to store sufficient quantities of packed RBCs. Therefore, extending the shelf-life of transfusible units and simplifying the storage logistics is critical to meet the blood demand in these regions.

The brine shrimp *Artemia franciscana*, also known as 'sea monkeys,' found in the Great Salt Lake in Utah, might offer a natural solution to these challenges. The encysted embryos of this organism can survive desiccation for decades, and upon rehydration, the animal resumes development. The encysted brine shrimp embryos can tolerate dehydration by utilizing molecular strategies, such as accumulating late embryogenesis abundant (LEA) proteins and the sugar trehalose [4]. This biocompatible sugar can preserve biological compounds in a glassy state at room temperature, and it thought to stabilize proteins by replacing the hydrogen bonds of water with its hydroxyl groups [5].

One approach to increase the shelf-life of blood is freeze-drying, which has already been successfully used to stabilize other blood components, such as platelets, for long-term storage at ambient temperatures [6]. Therefore, we investigate freeze-drying RBCs in a non-toxic solution of trehalose as a method for long-term stabilization. Lyophilization is a two-step process where RBCs in a buffer containing trehalose are frozen and then placed under a vacuum to sublimate the ice to obtain a dried product with the least amount of damage to cells possible.

I hypothesize that biochemical strategies enabling *A. franciscana* embryos to survive desiccation, such as the protective sugar trehalose, can increase the shelf-life of RBCs. Several studies have investigated the viability of lyophilized RBCs after rehydration. Still, most studies focused only on cell recovery without a detailed investigation into the physiological properties after rehydration [7] [8] [9]. Functional properties of the respiratory pigment hemoglobin after lyophilization and rehydration, which makes up the most of the RBC protein content, need further investigation.

Hemoglobin is the protein responsible for transferring oxygen from the lungs to the tissues by having oxygen bind reversibly to the iron in the heme group of the protein. For hemoglobin to bind oxygen, the iron atom must maintain an oxidation state of Fe^{2+}. Unfortunately, our research has shown that oxidative stress during the lyophilization process results in the oxidation of iron from the ferrous Fe^{2+} to the ferric $Fe^{3+,}$ forming methemoglobin, which cannot bind oxygen. Methemoglobin exists in insignificant amounts in the human body and is usually quickly reduced to oxyhemoglobin, which is the functional state of hemoglobin. However, considerable methemoglobin blood concentrations, resulting in methemoglobinemia, have been observed in some patients. One of the treatments for methemoglobinemia is supplementation with ascorbic acid [10].

Moreover, for hemoglobin to effectively transfer oxygen from the lungs to the tissues, it needs to be receptive to its allosteric regulators. Allosteric interactions are interaction on a site other than the active site on a protein and it has the effect of regulating the affinity of the active site via change in conformation. The ligand that binds at the allosteric site is called an allosteric regulator. The tetrameric protein is thought to exist in two allosteric conformations, a tout (T) state with low oxygen affinity and a relaxed (R) state with a higher affinity for oxygen. Negative-allosteric regulators, such as 2,3-bisphosphoglycerate (2,3-BPG), lower the oxygen affinity of the respiratory pigment and cause a right shift of the oxygen binding curve. The lower oxygen affinity of the pigment is generated by shifting the allosteric equilibrium of the protein towards the T state by predominantly binding to this conformation. Optimal oxygen loading at the lungs and release into the tissues results from allosteric modulation via 2,3-BPG [11].

Our current research aims to investigate the allosteric effect of 2,3-BPG on hemoglobin after the lyophilization of porcine RBCs to determine if rehydrated RBCs maintain physiological properties suitable for transfusion. Using the Blood Oxygen Binding System (BOBS), the partial pressure (the pressure exerted by a single gas in a mixture of gases in a definite volume) of O_2 at which 50% of hemoglobin is bound to oxygen can be determined ($P_{50}O_2$). In simple terms, this value represents the amount of oxygen required for 50% of the hemoglobin to be saturated with oxygen and is used as a measure of affinity. A rightward shift in the $P_{50}O_2$ means a decrease in oxygen affinity to hemoglobin and This study allows us to characterize the impact of modifications to the lyophilization and rehydration on the allosteric properties and regulation of porcine hemoglobin to develop a freeze-dried RBC product suitable for transfusion medicine.

Materials and Methods

Chemicals

Low endotoxin α, α-trehalose dihydrate was acquired from Pfanstiehl Inc. (Waukegan, IL). All other compounds were sourced from VWR (Radnor, PA) or MilliporeSigma (Burlington, MA) and were of the highest purity available. Water for solution preparation was endotoxin free and purchased from VWR (Radnor, PA).

Blood processing

Porcine blood was obtained from a local abattoir and collected in 50-100 IU heparin/mL. After decanting any formed clots and plasma, two washes with Dulbecco's phosphate-buffered saline (DPBS) were performed, and the washed red blood cells (RBCs) were collected by centrifugation at 600 g at 4 °C for 10 minutes. The final pellet was resuspended in an AS-3 (55 mM dextrose, 70 mM NaCl, 2.2 mM adenine, 2 mM citric acid, 20 mM sodium citrate, 23 mM monobasic sodium phosphate) blood storage solution and stored at 4 °C for a maximum of three weeks.

Oxidation determination

This work was done by me colleague Ethan Mills.

RBCs were diluted in 3x their volume of water and mixed at 4°C for 30 minutes. The RBC lysate was then centrifuged at 9000g for 15 minutes to remove membrane lipids and better facilitate filtration. The supernatant was removed from the sample (it may be necessary to

backlight the sample to dis tinguish the membrane lipids.) Further dilution (5-15x) was performed as necessary to facilitate flow through a 0.2μM filter. The sample was then forced by syringe through a 0.2μM filter. Dialysis was performed in 50mM Tris-Acetate Buffer pH 8.3 (Buffer A) overnight. The sample was then purified using FPLC with a Cepharose DEAE FF 5mL column. Column loading was performed in Buffer A and elution was done using 50mM Tris-Acetate pH 7.0 (Buffer B). The elution protocol used a linear gradient of 25-100% Buffer B eluted in 12 column volumes at 2mL/min flow rate. The eluted hemoglobin fraction was then dialyzed against DI water overnight. The sample was stored in water at -80°C until further use or set aside to undergo methemoglobin conversion. Samples dedicated to becoming methemoglobin were combined with 2-5mM ferric cyanide and mixed at 4°C for 30 minutes. Excess ferric cyanide was removed using a G-25 column. The 100% oxyhemoglobin (OxyHb) and 0% OxyHb samples were analyzed by spectrophotometer both to verify their OxyHb content and ascertain their concentration. Verification of OxyHb content was done by comparison to published spectra of 100% and 0% OxyHb. Concentration determination was used by examining absorbance at 577nm and 630nm for 100% and 0% OxyHb, respectively and using published extinction coefficients to derive concentration from these values. 25, 50, and 75% OxyHb samples were then prepared by combining the 100% and 0% OxyHb samples proportional to their concentration. For each data point 0, 25, 50, 75, and 100% nested N=2 and N=3. To verify that no ferric cyanide remained in the 0% OxyHb sample, one of the 75% samples in each trial was re-examined after one hour to verify its OxyHb content had not significantly changed. It was stored in 4°C during this time. The standard curve is shown (Fig. 1)

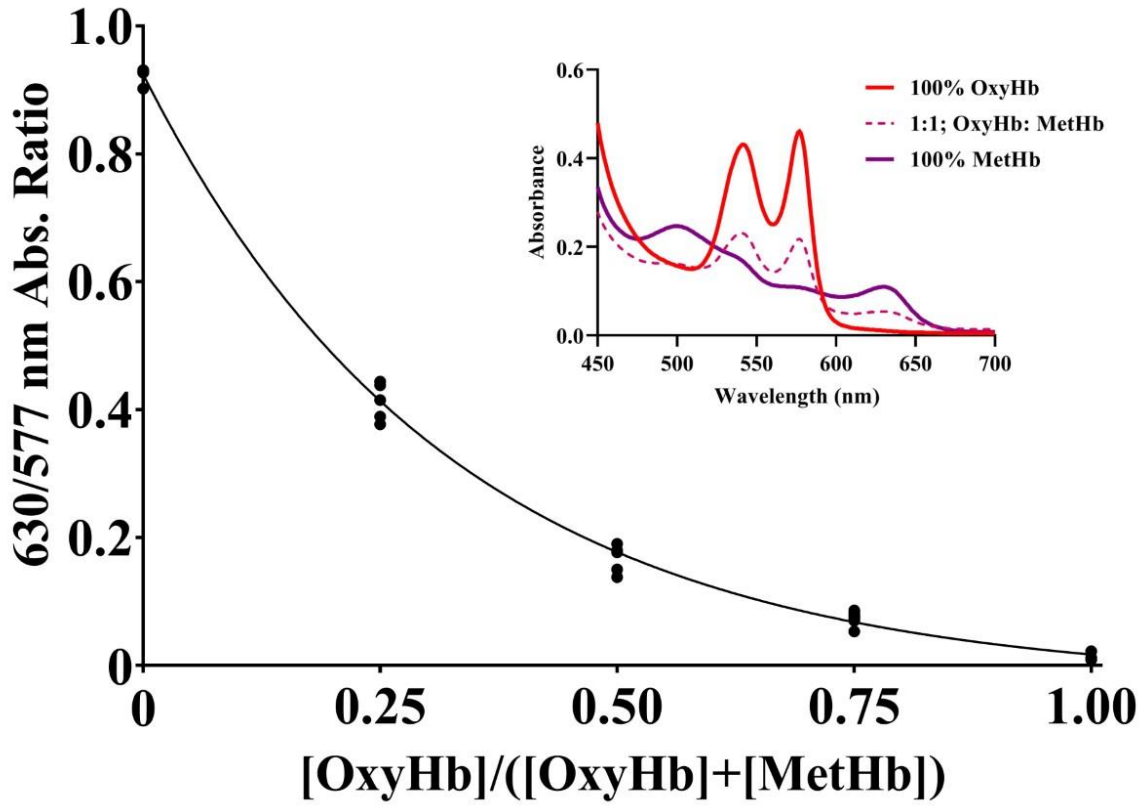

Fig. 1. The spectra of known proportions of OxyHb and MetHb were detrmined using the UV- vis. The ratio of 630 nm to 577 nm were measured to synthesize the standard curve used to calculate the amount of OxyHb in the rest of the study. (n = 3, 2-3 nested replicates)

Water content and oxidation

For each experimental trial, washed RBCs were enumerated before performing the experiment. In preparation for lyophilization, cells were resuspended in a solution composed of 300 mM trehalose, 30 mM NaCl, and 20 mM HEPES-NaOH, pH 7.1 at a concentration of 2.5 x 10^9 RBCs/mL and transferred to 1.5 mL traded centrifuge tube in 0.5 mL aliquots.

Samples were snap-frozen in liquid nitrogen and then transferred to a Labconco™ FreeZone™ Triad Freeze Dryer (Kansas City, MO). The shelf temperature of the lyophilizer was set to - 50 °C for 2 days for the primary drying step, followed by 30 °C for 1 day for the secondary

drying step. Following lyophilization, the samples were stored in a desiccation chamber over anhydrous $CaSO_4$ (Drierite) until experiments were performed.

Salt-isotherms (moisture sorption isotherms) yielding defined humidity were prepared using saturated NaCl solution in air-tight desiccation chambers. The humidity in these chambers at room temperature is 75% [12]. The mass of the samples was measured before transferring them to the chambers and after exposure to 75% humidity for times ranging from 30 minutes to 2 days. Moreover, an analytical balance was used to calculate the change in mass of each sample in that range to deduce the water content in each sample. The water content was recorded as grams of water per grams of dry weight, (gH_2O/gdw). A coulometric Karl-Fischer titrator was used to detriment the baseline water content of the samples after lyophilization by transferring exact amounts of the dry samples to the titration chamber.

Samples were rehydrated in 450 µL of 20% w/w dextran 40,000 and diluted 10 x with water before centrifugation at 1500 *g* at 4 °C for 5 minutes. The supernatant obtained after rehydration of RBCs was diluted in 100 mM NaCl and 100 mM HEPES, pH 7.1. Spectral analysis was performed using a Shimadzu UV-1800 UV-VIS spectrophotometer (Kyoto, Japan) with scans from λ = 750 - 250 nm to determine the presence of methemoglobin in the samples. The absorbance values at λ = 630 nm and 577 nm were used to determine the amount of oxyhemoglobin relative to methemoglobin.

Impact of storage condition on methemoglobin formation.

In preparation for lyophilization, porcine RBCs were suspended in a solution composed of 300 mM trehalose, 100 mM NaCl, and 20 mM HEPES-NaOH, pH 7.1. An addition of 6 mM ascorbic acid was added to samples Lyophilized with ascorbic acid. Samples were snap-frozen in liquid

nitrogen and then transferred to the lyophilizer. Each sample contains 0.5 mL of 2.5 x 10^9 RBCs/mL in its respective lyophilization buffer. RBCs in the freeze dryer were located above the cooling shelf, except for the vacuum glass vials, which were placed directly on the temperature-controlled shelf. The lyophilized RBCs were stored under 0% humidity at room temperature. Dried RBC samples were rehydrated with 450 µL of 20% w/v dextran 40,000 solution. RBCs were analyzed for up to 10 weeks after lyophilization. Before analysis was performed, centrifuged at 1500 *g*. UV-VIS spectral analysis of hemoglobin was performed in 100 mM NaCl and 100 mM HEPES-NaOH, pH 7.1 using a Shimadzu UV-1800 UV-VIS spectrophotometer, and the values at $\lambda = 630$ nm and $\lambda = 577$ nm were used to determine the amount of OxyHb, relative to MetHb.

Effect of lyophilization and rehydration with 6 mM ascorbic acid

In preparation for lyophilization, porcine RBCs were suspended in a solution composed of 300 mM trehalose, 100 mM NaCl, and 20 mM HEPES-NaOH, pH 7.1. An addition of 6 mM ascorbic acid was added to samples Lyophilized with ascorbic acid. Samples were snap-frozen in liquid nitrogen and then transferred to the lyophilizer. Each sample contains 0.5 mL of 2.5 x 10^9 RBCs/mL in its respective lyophilization buffer. RBCs in the freeze dryer were located above the cooling shelf, except for the vacuum glass vials, which were placed directly on the temperature-controlled shelf. The lyophilized RBCs were stored under 0% humidity at room temperature. Dried RBCs were then rehydrated with either 450 µL of 20% w/v dextran 40,000 solution for samples lyophilized with ascorbic acid or 450 µL of 20% w/v dextran 40,000 supplemented with 6 mM ascorbic acid for samples lyophilized without ascorbic acid. Samples were analyzed 0 min, 30 min, and 60 min after rehydration. Before analysis was performed, samples were diluted 1:9 with water, centrifuged at 1500 *g* for 5 minutes. UV-VIS spectral analysis of hemoglobin was performed in

100 mM NaCl and 100 mM HEPES-NaOH, pH 7.1 using a Shimadzu UV-1800 UV-VIS spectrophotometer, and the values at $\lambda = 630$ nm and $\lambda = 577$ nm were used to determine the amount of OxyHb, relative to MetHb.

Hemoglobin Oxygen-Binding Properties

RBCs from the storage buffer were diluted to 2.5 x 10^9 RBCs/mL in the respective lyophilization buffer (24 mM of glucose, 0.4 mM of adenine, 33 mM of NaCl, 8.9 mM of mannitol, 100 mM of trehalose, 15% w/v dextran 40,000, 2.5% w/v BSA, 6.6 mM potassium phosphate, pH 7.2. Samples at a volume of 0.5 mL are then frozen at a rate of -1 °C per minute from 23 °C to -80 °C and transferred to a Labconco FreeZone Triad Freeze Dryer for 3 days. For the first two days, the shelf temperature of the freeze-dryer is maintained at -40 °C for the primary drying step. On the third day, the shelf temperature is raised to 25 °C for the secondary drying step.

The lyophilized samples were stored over anhydrous Drierite to maintain 0% humidity at room temperature. Samples were rehydrated with 0.5 mL of 20% w/v dextran 40,000, 6 mM ascorbic acid, and 20 mM HEPES-NaOH, pH 5.1. An aliquot of 350 µL of the sample is then centrifuged at 9,600 *g* for 15 min, and the supernatant (305 µL) is removed, followed by adding 5 µL of 1 M NaCl, 1 M HEPES-NaOH, pH 7.1 to 45 µL resuspended pellet. Moreover, 50 µL of 20 mM 2,3-BPG, 100 mM NaCl and 100 mM HEPES-NaOH, pH 7.1 is added to the pellet so that the final product will be 100 µL of RBCs with 10 mM 2,3-BPG, 100 mM NaCl and 100 mM HEPES-NaOH, pH 7.1. Aliquots of 1.5 µL were analyzed using the BOBS to measure the $P_{50}O_2$ of the samples. An oxygen binding curve was obtained for each sample (Fig. 2)

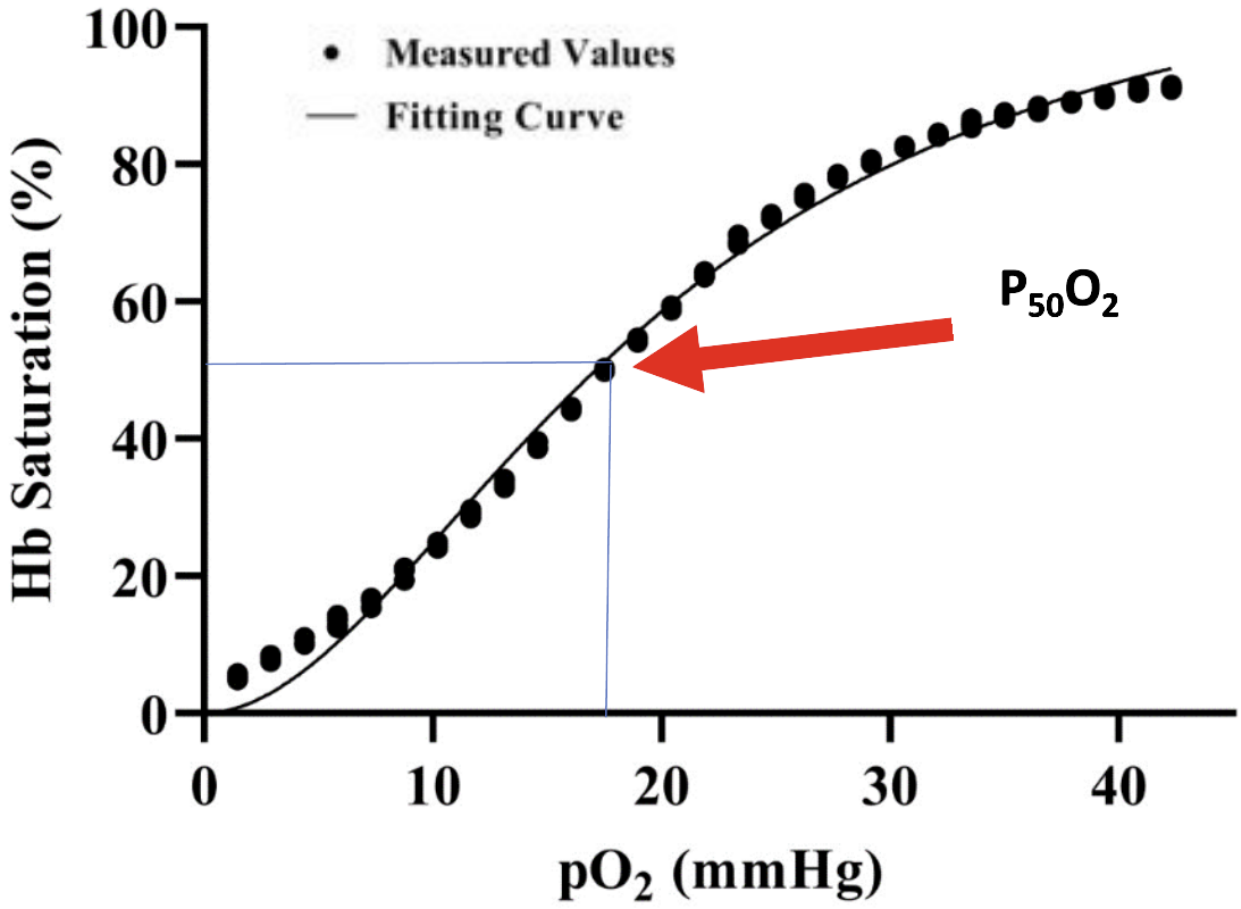

Fig. 2. Oxygen binding curve was obtained using the BOBS. The BOBS software was used to calculate the $P_{50}O_2$ of the samples.

Statistical analyses

Data were analyzed using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA) and GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA).

Results

Effect of humidity on hemoglobin Oxidation

Lyophilized RBCs were stored at 75% humidity chamber to pre-hydrate the dried samples. The effect of pre-hydration on lyophilized RBCs was evaluated to determine if spontaneous rehydration can preserve the functionality of hemoglobin. The fraction of OxyHB decreases with increased water content with a correlation coefficient of $r^2 = 0.99$ (Fig. 3A). Furthermore, as the water content in the lyophilized samples increased, the volume of the samples decreased, and the color of the freeze-dried product changed from light red to black (Fig. 3B).

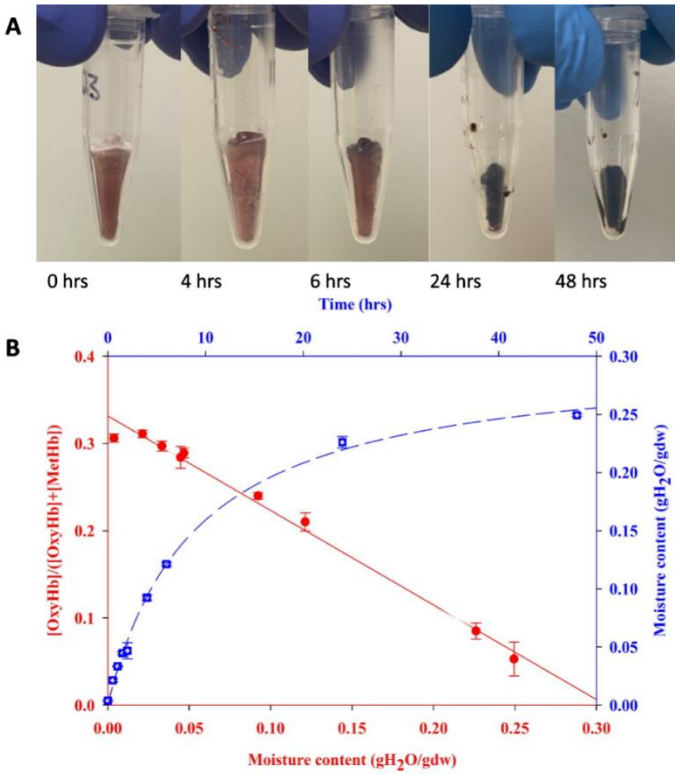

Fig. 3. Pre-hydration utilizing salt-isotherms at 75% humidity increases lyophilized RBCs water content, which is inversely correlated with the fraction of OxyHb **A** The physical changes of the samples with time at 75% humidity. **B** • Proportion of OxyHb in respect to water content. ◘ Moisture content in relation to time at 75% humidity. (n = 3)

Impact of storage condition on methemoglobin formation.

Three storge conditions were assessed over the 10 weeks to determine the optimal storage method for lyophilized RBCs. Based on Two-way ANOVA analysis there is a significant difference based on storage conditions and time, in which the amount of OxyHB decreases over time for all storage

conditions (Fig. 4). Based on Tukey post-hoc analysis we can infer that supplementing the samples with ascorbic acid significantly reduces the amount of oxidation.

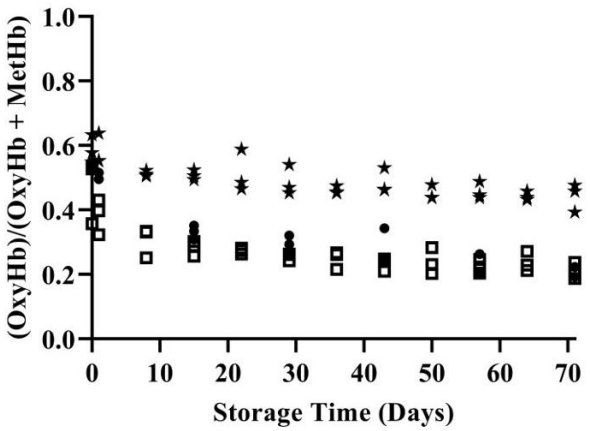

Fig. 4. The fraction of OxyHb over 10 weeks of storage. □ 300 mM Trehalose lyophilization buffer. ★ 300 mM Trehalose with 6 mM Ascorbic acid lyophilization buffer. • 300 mM Trehalose lyophilization buffer stored under vacuum. Based on the data gathered, we detected a storage time and storage condition effect. (n = 3)

Effect of lyophilization and rehydration with 6 mM ascorbic acid

To investigate weather ascorbic acid prevents or reverse hemoglobin oxidation, RBCs samples were supplemented with ascorbic acid before or after lyophilization. Based on repeated measures ANOVA There is a significant difference in hemoglobin reduction over time after rehydration, but no effect was detected at which step 6 mM ascorbic acid was added (Fig. 5).

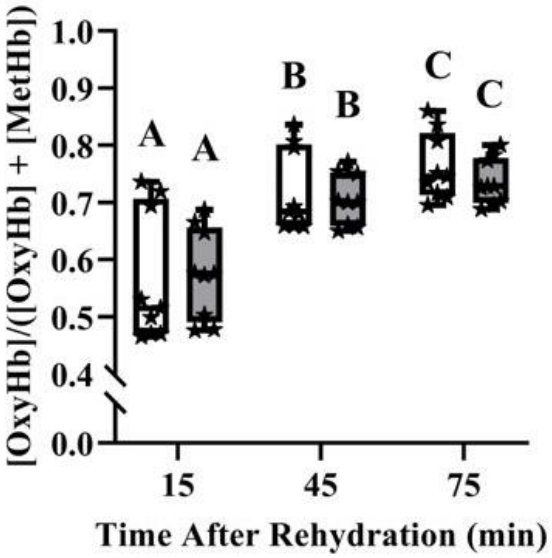

Fig. 5. Fraction of OxyHb present in RBCs after rehydration with 6 mM ascorbic acid present in the: lyophilization buffer (**White**), or rehydration buffer (**Gray**). (n = 3, 3 nested replicates)

Hemoglobin Oxygen-Binding Properties

Oxygen binding properties of hemoglobin within RBCs were studied to determine if degradation occurs to intracellular hemoglobin after lyophilization and rehydration. Hemoglobin was analyzed before and after lyophilization and rehydration. Based on two-way ANOVA no effect in the allostatic regulation of hemoglobin was deducted on the dry product. (Table. 1)

Condition	**Additive**	**P_{50} O_2 [mmHg]**	**n_H**
Before Lyophilization	No Additive	10.16 ± 1.02	2.08 ± 0.18
	Ascorbic Acid	14.74 ± 0.27	1.73 ± 0.11
	Ascorbic Acid + 2,3 BPG	24.36 ± 2.70	1.96 ± 0.075
After Rehydration	No additive	8.09 ± 1.06	1.81 ± 0.23
	Ascorbic Acid	18.81 ± 4.84	1.55 ± 0.07
	Ascorbic Acid + 2,3 BPG	26.75 ± 9.54	1.64 ± 0.06

Table 1. Oxygen-binding properties of intracellular hemoglobin before and after lyophilization and rehydration of RBCs. No significant difference was detectable between additive conditions before and after lyophilization and rehydration ($p > 0.05$; $n = 4 – 9$).

Discussion:

Lyophilized RBCs have the potential to save countless lives by extending the shelf life of blood and making it more accessible. Hemoglobin oxidation is one of the challenges that must be addressed to have dried RBCs that are suitable for transfusions.

The result of this study suggests that lyophilized RBCs are hygroscopic to the point where they can absorb a measurable amount of moisture from the air. Furthermore, the hygroscopicity of dried RBCs decreases with increasing water content (Fig.3B). This pre-hydration results in decrease in volume and increase in mass (Fig.3A). Under these conditions hemoglobin converts to non-functional state termed met hemoglobin. These results were not consistent with the positive impact of pre-hydration found in platelets [13]. The exact mechanism of hemoglobin oxidation resulting from the pre-hydration of dried RBCs is currently under investigation.

Moreover, there was no significant difference between samples that were exposed to oxygen during storage at 0% humidity and those that were stored under a vacuum (Fig. 4). This suggests that autooxidation – the spontaneous oxidation of compounds as results of oxygen exposure – is not the primary oxidation mechanism hemoglobin in lyophilized RBCs [14]. Further research is needed to investigate the oxidation mechanism of hemoglobin.

Adding ascorbic acid showed a promising result with regards to hemoglobin oxidation. Samples that were supplemented with ascorbic acid, showed a significant decrease in the rate of oxidation during storage compared with those that were not (Fig. 4). Ascorbic acid was able to reduce oxidized hemoglobin after rehydration. Moreover, there was not a significant difference between samples that were lyophilized and those that were rehydrated with ascorbic acid, meaning that ascorbic acid retains its ability to reduce hemoglobin after lyophilization (Fig. 5). These results are

consistent with previous studies that shows the reduction potential of ascorbic acid on the amount of MetHb, although this effect is dependent on the concentration of ascorbic acid [15]. Further research needs to be performed to assess the limits of ascorbic acid stability in lyophilized RBCs, since it is known that ascorbic acid is sensitive to both air and oxygen [16].

It was demonstrated that the allosteric regulation of hemoglobin was preserved after lyophilization (Table 1). The $P_{50}O_2$ of hemoglobin and the effect of ascorbic acid on the allosteric regulation in this study are inconsistent with the existing literature, though previous experiment has used different methodology [15 17].

Further research needs to be performed to determine exact mechanism of hemoglobin reduction and the possibility of activating the enzymatic reduction pathways of MetHB [18]. More optimization is needed to reach hemoglobin oxidation levels that are suitable for transfusion.

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