Rehydration Outcomes for Freeze-Dried Red Blood Cells in Reduced Gravity

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Abbreviations

RBC, Red blood cell; PBS, Phosphate buffered saline

Keywords: Parabolic flight, freeze-dried RBCs, 0 *g*, fluid dynamics, transfusion, anhydrobiosis

Abstract

Medical planning for space exploration is based on the "floating" blood bank model to store life-saving red blood cells (RBCs) for emergencies. The "floating" blood bank approach is not sufficient in cases where multiple crewmembers are affected by space anemia. In these situations, long-term preserved RBCs will be vital to guarantee the health and safety of crew members. Transfusable RBC units can only be refrigerated for 42 days or frozen at -80 °C. However, storing frozen RBCs at -80 °C is challenging during the confined condition of long-duration space flight. Freeze-dried, viable RBCs would be an appropriate alternative because they can be stored without cooling, are predicted to have a shelf-life of years, and could be transfused immediately after rehydration. This study explores if freeze-dried RBCs can be rehydrated and transfused in reduced gravity with similar outcomes in recovery as observed at Earth gravity. Experiments analyzing freeze-dried RBC recoveries, rehydration fluid dynamics, and transfusion flow rates were analyzed utilizing an experimental glovebox in simulated 0 *g* during parabolic flights. RBC recoveries and rehydration fluid dynamics for volumes of 5 mL and 10 mL were the same in simulated 0 *g* compared to results obtained at 1 *g*. A clinically acceptable range of flow rates for slow intravenous infusion and rapid fluid resuscitation was possible with the simple augmentation of a hand-pumped clinical pressure bag around a unit of rehydrated RBCs. The results demonstrate the potential feasibility of using freeze-dried cells for healthcare during deep-space exploration.

1. Introduction

Blood transfusions are one of the most critical medical procedures conducted. Over 10 million red blood cell (RBC) units are transfused yearly in the United States [1]. Unfortunately, it is challenging to maintain a sufficient supply of RBCs for transfusion medicine. Blood donations must carefully match the demand for transfusions, and a reliable temperature-sensitive process is required to sustain the RBCs during storage. Maintaining a blood supply can be challenging in extreme conditions on Earth, and even with the many preparations and precautions taken for space flight, life-threatening situations are still possible during a mission [2]. Current storage methods for RBCs require refrigeration at 4 °C or frozen storage at -80 °C. Refrigerated RBCs can only be stored for 42 days, leading to supply shortages [3]. Frozen RBCs are loaded with glycerol in a complex procedure before freezing, allowing them to be stored for up to 10 years. However, the glycerol must be carefully removed with a series of washing steps after thawing, making the process sensitive, time-consuming, and resource constraining, which poses challenges for aerospace engineers when designing spacecraft [4].

Space anemia is the leading motivation for the development of transfusion medicine in reduced gravity. Anemia is a condition that develops when an individual has lower-than-normal amounts of healthy RBCs. Studies indicate a reduction in plasma volume while in 0 *g* leading to an initial increase in hematocrit. To counteract the sudden increase in hematocrit the body lowers the production and increases the destruction of RBCs to return to pre-exposure concentrations [5]. While anemia is becoming less of a concern while traveling in outer space, it is speculated to be a severe problem when reaching non-Earth destinations when plasma volumes increase. Many astronauts experience anemia after returning to Earth due to increased plasma volume and reduced RBC production while in 0 *g* [6; 7]. This is easily treatable on Earth with RBC transfusions and

non-mission personnel available to perform precise tasks. Still, reaching non-Earth destinations could become a pressing issue without access to transfusable RBCs leading to dizziness, weakness, and limited endurance during crucial mission objectives.

Astronauts also have a high risk of life-threatening hemorrhage during a space flight due to a traumatic injury or other medical conditions. Current medical planning for space exploration incorporates the "floating" blood banks model, based on the terrestrial walking blood bank protocols used in combat zones since World War II and other austere environments worldwide [8]. The implementation of a "floating" blood bank describes the concept of matching blood types of crew members to be able to perform direct person-to-person whole blood transfusion in cases of emergencies. The effects of cosmic radiation on RBC and hemoglobin production are still under investigation [6]. The "floating" blood bank protocol would not be feasible if multiple crewmembers are affected by space anemia. Significant decreases in hematocrit values during an extended space flight, where injury, illness, and partial malfunctions of the life support systems are possible, could be detrimental to an astronaut's health and performance [9; 10]. There is currently no reasonable way to store large quantities of transfusable RBCs in a freezer or refrigerator during an extended space mission.

There have been significant research efforts to address these challenges by focusing on developing artificial blood substitutes. Despite substantial investments, no synthetic blood products are available clinically, and patients in clinical trials experienced cardiovascular complications from hemoglobin-based artificial blood products [11; 12]. Recently, there have been efforts to generate stem cell-derived RBCs, but these cells still face the same storage challenges as natural RBCs [13]. Issues such as oxidative damage to RBC membranes during refrigeration could be reduced and possibly eliminated if RBCs were stored at ambient temperatures in a desiccated

state. Animals that have developed a natural propensity to survive for years in a desiccated state (termed “anhydrobiosis”), have inspired researchers to engineer desiccation-tolerant human RBCs [14; 15]. Dry preservation of RBCs would increase their shelf life and enable transfusions in places with highly challenging environments. These environments include but are not limited to, military operations, developing countries, remote medical centers, and space exploration missions.

Exceptional animals in multiple phyla (e.g., Tardigrada, Nematoda, Arthropoda, and Rotifera) and plants (resurrection plants and orthodox seeds) can survive extreme desiccation for decades if not millennia after the accumulation of protective osmolytes such as sucrose and trehalose [16; 17; 18; 19; 20; 21]. We have been developing and optimizing methods to dehydrate RBCs for long-term storage using the cell impermeant sugar trehalose. This process includes preloading the cells with the sugar through a technique involving ultrasound-mediated transmembrane delivery (“sonoporation”) [15]. Two different freezing methods, bulk-freezing and spray-freezing, were investigated to determine their influence on rehydration outcomes of freeze-dried RBCs in 1 *g* vs. 0 *g*. Freeze-dried RBCs have not yet entered clinical trials and optimization of drying and rehydration of the cells must be performed beforehand. This study is a proof-of-principle investigation and covers RBC rehydration research from five parabolic flights, consisting of 150 total parabolas which demonstrated the potential feasibility of utilizing desiccated RBCs stored at ambient temperature for space exploration.

2. Materials and Methods

2.1 Chemicals

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

2.2 Micro- and Hypergravity Simulation

Microgravity was simulated by five parabolic flights provided by Zero Gravity Corporation (Exploration Park, FL). Flight investigators boarded a modified Boeing 727, which flew thirty parabolic flight paths to simulate 0 *g* for 14 – 21 seconds per parabola. Between each parabola hypergravity was experienced with an average force of 1.8 *g* (Fig. 1). Gravitational force was measured with a piezoresistive accelerometer model 2262-25 (Endevco Corp.) (Fig S1).

2.3 Experimental Glove Box

All experiments were performed in a custom glovebox to provide secondary containment (Fig 2), and camera mounts on the canopy recorded the experiments. Three pairs of arm access ports permitted interaction with the experimental setup, and external electrical connectors in the glovebox received power from the aircraft to distribute if needed. Four personnel from the research team wore hands-free headsets to communicate during the parabolic flight experiments. Researchers used foot straps next to the glovebox to restrain themselves during microgravity periods.

2.4 Fluid Dynamic Analysis

The fluid dynamics inside a syringe rehydration system were assessed using a camera inside the glovebox (Fig. 3A & 3B). A fluorescein solution of 100 mg/L in water was used to rehydrate the desiccated RBCs. The camera recorded videos to monitor an injection and withdrawal repeated three times of the fluorescein solution into the dehydrated RBC samples throughout each microgravity and normal gravity trial (Fig. 3C). The software MATLAB (MathWorks, Natick, MA) was utilized to analyze the two-dimensional video frames and quantify the maximum percentage of the blood bag filled with fluorescein (green) solution during the three injections and withdrawals while rehydrating 5 mL and 10 mL of spray dried RBCs at 1 *g* and 0 *g* (Fig. 3C).

2.5 Porcine RBC Collection

Porcine blood was acquired from a local abattoir, JBS USA (Louisville, KY), and collected in tubes containing a 50-100 units/mL heparin solution. The whole blood was pelleted at 600 *g* for 10 min using a Centrifuge 5804R (Eppendorf, Hamburg, Germany). After centrifugation, the supernatant of the solution was decanted, and the pellet was resuspended in phosphate buffered saline (PBS) (Cytiva, Marlborough. MA). The centrifugation-based RBC washing process was performed three times but with the final resuspension to 50 – 60 % hematocrit in the FDA-approved RBC storage solution, Additive Solution-3 (70 mM NaCl, 2 mM citric acid, 23 mM Na_3-citrate, 2 mM adenine, 55 mM dextrose, 23 mM NaH_2PO_4, pH 5.8). Washed RBCs were stored for no longer than seven days at 4 °C before freeze-drying.

2.6 Trehalose Loading

RBCs were diluted to 250 million RBCs/mL in a solution containing 300 mM trehalose, 100 mM NaCl, and 20 mM HEPES, pH 7.1. RBCs were loaded with the sugar trehalose using microfluidic sonoporation as previously described [15]. After loading the cells, the RBCs were centrifuged, decanted, and then resuspended at a concentration of ~9 billion RBCs/mL in a solution composed of 300 mM trehalose, 100 mM NaCl, 6% w/v ficoll 400, 1% v/v ethanol, and 20 mM HEPES, pH 7.1.

2.7 Bulk Freeze-Drying

A volume of 25 mL of prepared RBCs were placed into each cryogenic glass jar. The RBCs were then rapidly frozen by placing the jars into liquid nitrogen, and the jars were transferred into a FreeZone Triad freeze-dryer (Labconco Corporation, Kansas City, MO). Dried RBC cakes that were bulk-frozen can be seen in Fig 4A. After the samples were dried, they were transferred into 100-mL dual-chamber bags (Fujifilm, Tokyo, Japan) (Fig. 5), heat-sealed, and stored over anhydrous $CaSO_4$ (DriRite, Xenia, OH) until rehydrated.

2.8 Spray Freeze-Drying

A 25 mL volume of prepared RBCs was sprayed into a 125 mL glass jar containing stirred liquid nitrogen using a peristaltic pump and a nasal aerosolizer. This approach leads to individual droplets with volumes of less than 10 μL. These small volumes have significantly different heat transfer and heat dissipation kinetics during freezing compared to bulk volumes of 25 mL. The RBCs were then dried and stored the same as described in section 2.7. Spray freeze-dried RBCs can be visualized in Fig. 4B.

2.9 RBC Recovery

Freeze-dried RBCs were rehydrated with 100 mL of PBS at 1 g and simulated 0 g by rupturing the dual-chamber bag membrane that separates the rehydration solution and freeze-dried RBCs then alternating the application of pressure to each chamber until no visible solid particles were observed. Rehydrated RBC concentrations were enumerated using a Vetscan HM2 Hematology Analyzer (Abaxis Inc., Union City, CA). RBC recovery percentages were determined by dividing the total amount of RBCs after rehydration by the total before drying and multiplying the fraction by 100%.

2.10 *Scanning Electron Microscopy*

The dry RBC products were scanned and micrographed using an Apreo C LoVac Field Emission Scanning Electron Microscope (Thermo Fisher, Waltham, MA). Samples were sputter-coated with gold-palladium before imaging.

2.11 Transfusion Studies

A unit (~450 mL) of rehydrated RBCs was transfused into a training mannequin forearm vein through a 17-gauge butterfly needle IV set with the flow resistor fully opened for starting a fluid resuscitation scenario. The steady-state flow rate for each acceleration condition was measured by an ultrasonic transit-time flow probe (Transonic Systems, Ithaca, NY) incorporated into the transfusion line (Fig. 6). Infusion flow measurements were performed in micro- and hypergravity while a pressure sleeve around a unit of rehydrated RBCs was inflated to pressures of 0, 150, and 300 mmHg.

196 *2.12 Statistical Analyses*

197 Data were analyzed with two-way ANOVA tests using GraphPad Prism 9 (Graphstats
198 Technologies, Bengaluru, India) employing a Tukey-Kramer post hoc analysis. Bars on bar graphs
199 represent averages, and error bars represent standard deviations.

3. Results

3.1 Rehydration Fluid Dynamics in 1 g vs. 0 g

When rehydrating a powdered sample on Earth, the standard method is to simply pour the rehydration solution into the container holding the compound of interest. However, this procedure does not apply to a 0 *g* environment because pouring is a gravity-mediated process. To overcome this challenge, a rehydration procedure was developed that utilized increased pressures generated by compressing a syringe (Fig. 3B). A significant concern with the syringe rehydration system was that the pressure from the syringe would not create a large enough force to fill the blood bag at 0 *g*. Two volumes of 5 mL and 10 mL were examined at 1 *g* and in simulated microgravity. Analysis of the two-dimensional rehydration footage revealed no significant difference in the percentage of the bag that was filled during the rehydration at 1 *g* and simulated 0 *g* for both volumes used ($p > 0.05$, $n = 10$) (Fig. 7). The percentage of filling the blood bags did not reach 100 % in any trial because blood bags with a max volume of 25 mL were employed for the study.

3.2 Freeze-Dried RBC Recovery After Rehydration at 0 g and 1 g

The short shelf-life of RBCs poses problems for astronauts embarking on interplanetary travel, which would take about 21 months for a roundtrip to our nearest planet Mars. A potential solution is dried RBCs with a shelf-life of years at room temperature and can be rehydrated when needed without a time-consuming preparation process. Studies conducted in simulated microgravity were performed to determine if freeze-dried RBC would have a similar rehydration outcome in reduced gravity compared to 1 *g*. Two different types of freeze-dried RBC products were compared to investigate if the shape and characteristics of the product influence cell viability after rehydration in reduced gravity. Bulk freeze-drying, a standard method for lyophilizing biologicals, was

compared to spray freeze-drying (freeze-dried products can be viewed in Fig. 4). Scanning electron micrographs showed that the microstructure of the spray freeze-dried product was especially porous (more pores/unit area) compared to the layered structure of the bulk freeze-dried product (Fig. 8). Additionally, the RBCs were observed to be less impacted by hyperosmotic shock in the spray freeze-dried vs. the bulk freeze-dried condition (Fig. 8 & 9). Spray freeze-dried RBC droplets had a distribution of diameters ranging from ~ 0.05 – 1.5 mm (Fig. 9). After the cells were rehydrated in 1 *g* and simulated 0 *g* a significant difference in RBC recovery was detectable between the bulk and spray freeze-dried samples ($p < 0.05$, $n = 6$) (Fig. 10). Spray freeze-dried RBCs samples were on average recovered at ~30% higher numbers than bulk freeze-dried RBCs samples. No significant difference was detected when comparing RBC recoveries after rehydration at 1 *g* versus simulated microgravity ($p > 0.05$, $n = 3 – 6$) (Fig. 10).

3.3 RBC Transfusion Studies

The ability to transfuse rehydrated red blood cells was demonstrated inflight for a range of accelerations including 0 *g*, 1 *g*, and 1.8 *g*. Unaugmented infusion flow rate dependency on hydrostatic pressure is apparent by the absence of flow in 0 g and increasing flow with increased acceleration level. Dramatic flow augmentation by the use of a standard, hand-pumped, clinical pressure sleeve around the unit of rehydrated red blood cells was observed at all levels of acceleration (Fig. 11). This demonstration provided consistent evidence that a clinically acceptable range of flow rates for slow intravenous infusion or rapid fluid resuscitation was possible to achieve with the simple augmentation of a hand-pumped, clinical pressure sleeve around the unit of blood.

4. Discussion

Uncontrolled hemorrhage within hours of injury is the leading cause of preventable deaths worldwide [22]. Current storage practices for transfusable RBCs are impractical aboard a spacecraft because of their limited shelf-life and cold-chain requirements. Freeze-dried RBCs are a sensible alternative storage strategy to standard packed RBCs because they are anticipated to have an increased shelf life (e.g. 3 – 5 years), could be stockpiled, and stored at room temperature [23]. In the current study, the fluid dynamics of the rehydration solution and the yield of morphologically intact freeze-dried RBCs after rehydration were compared at 1 *g* and simulated microgravity. Differences in freeze-dried RBC rehydration outcomes at various gravitational forces were undetectable in both fluid dynamics and RBC recovery studies (Fig. 7 and 11). These results indicate that rehydrating freeze-dried RBCs on board a spacecraft on an interplanetary mission can yield morphologically intact cells and potentially be used to stop hemorrhagic shock and treat anemia. However, improvements in the total yield of RBCs are still needed to develop a transfusible unit, and studies aimed at reducing hemolysis after rehydration to below 5% are currently ongoing. For example, as shown in Fig. 10, a change in the volume at which the cells were frozen can considerably impact RBC yields after rehydration. This result is not surprising considering the large release of thermal energy during ice formation[24]. Tiny droplets in direct contact with liquid nitrogen will have substantially different heat dissipation compared to large bulk samples in glass vials, leading to less uniform freezing of the RBCs for the latter [25]. Furthermore, it is hypothesized that the porosity of the dried products has a significant impact on the rehydration kinetics, which may facilitate the variations in cell yields between the two freeze-drying conditions (Fig. 8).

Transfusion studies were performed to verify that RBCs rehydrated in reduced gravity could be administered into a patient. RBC transfusion flow rates were obtainable in reduced gravity with a pressure bag. A clinically acceptable range of flow rates for slow intravenous infusion or rapid fluid resuscitation was possible with the simple augmentation of a standard hand-pumped clinical pressure bag around a unit of blood (Fig. 5 and 11) [26].

A limitation of the research performed was that RBCs rehydrated at 0 *g* could not be analyzed immediately due to the short duration of reduced gravity provided by parabolic flights (less than 30 s). RBC samples rehydrated at 0 *g* experienced 1 – 2 hours at 1 *g* prior to recovery measurements. Ideally, rehydrated RBCs would be analyzed in reduced gravity to verify that the increased gravitational force experienced directly after the rehydration does not affect cell health. Performing the experiments and analysis on the International Space Station or during longer simulated microgravity of a suborbital flight would yield more robust evidence of the practicality of using freeze-dried RBCs in reduced gravity.

5. Conclusion

Overall, the present work demonstrates the feasibility of rehydrating freeze-dried RBC products in simulated microgravity which we expect would hold true for environments experienced in outer space. However, the overall recovery of freeze-dried RBCs still needs to be improved. Additionally, the physiological effectiveness of hemoglobin's oxygen binding capabilities after freeze-drying needs to be analyzed, and the retention of hemoglobin within RBCs needs to be characterized. Despite these limitations we observed no negative impact of microgravity on the yield of rehydrated RBCs compared to samples processed at ambient gravity. This is an important finding to move forward in our attempt to develop transfusion products that can be employed during space missions.

5. Acknowledgements

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6. Figures

Fig. 1

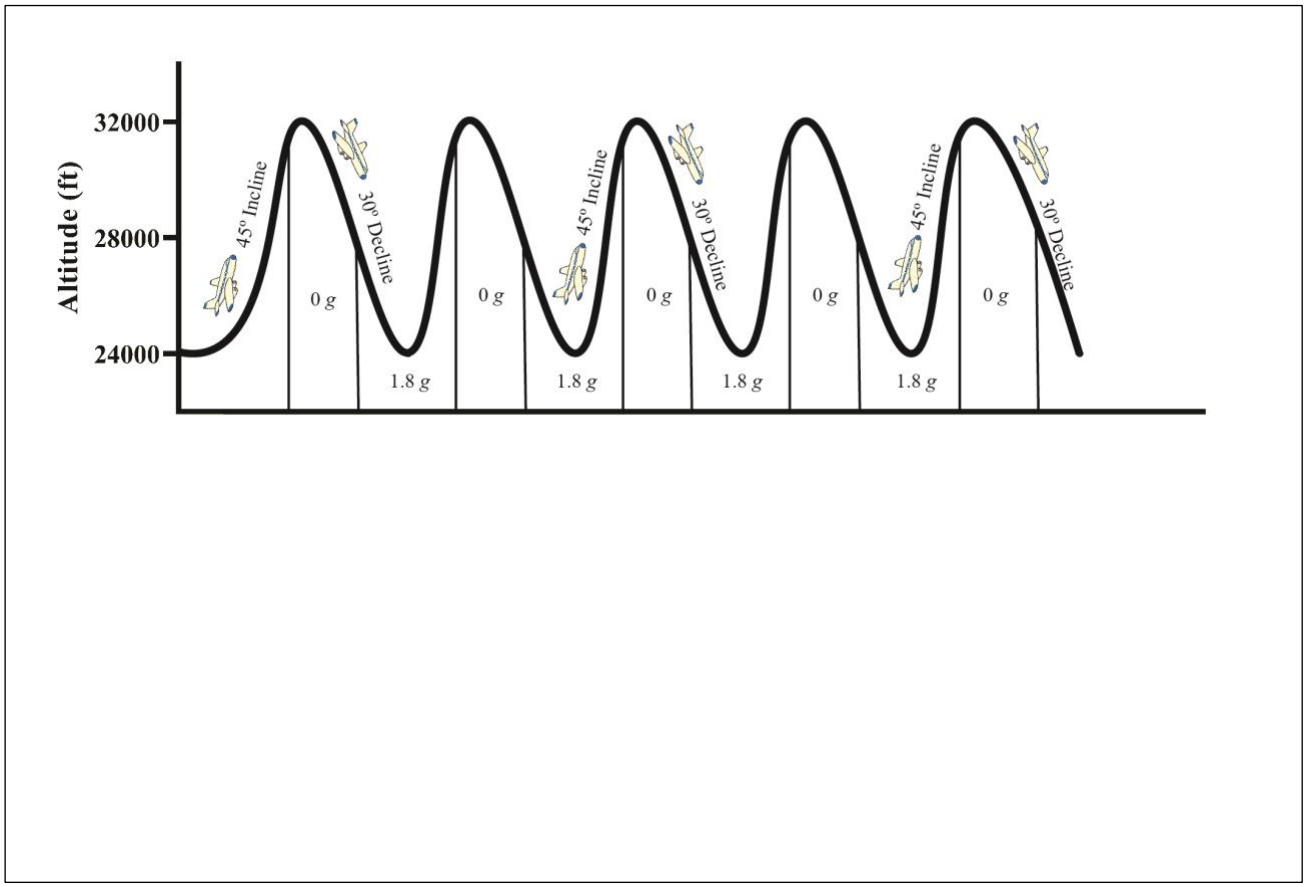

Fig. 1. Flight pattern taken by the modified Boeing 727 to produce microgravity. Five parabolic flight patterns were flown sequentially to produce five ~20-second increments of simulated 0 *g*. This flight pattern was performed six times per flight to produce a total of 30 increments of simulate 0 *g*.

Fig. 2

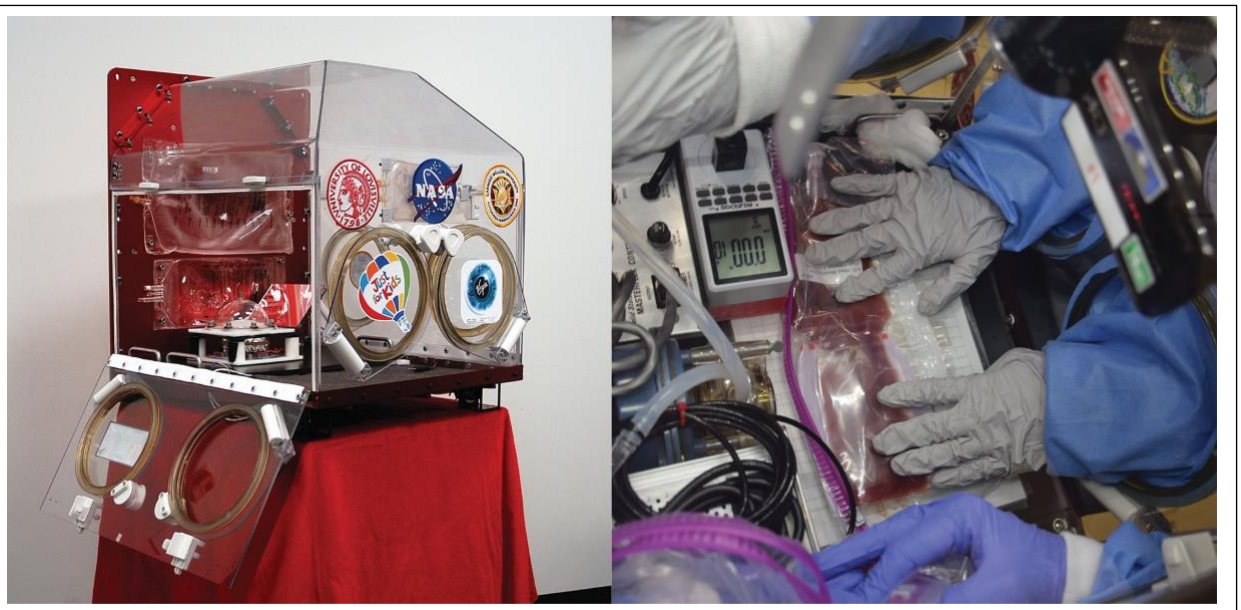

Fig. 2. The experimental glovebox utilized during the parabolic flights.

Fig. 3

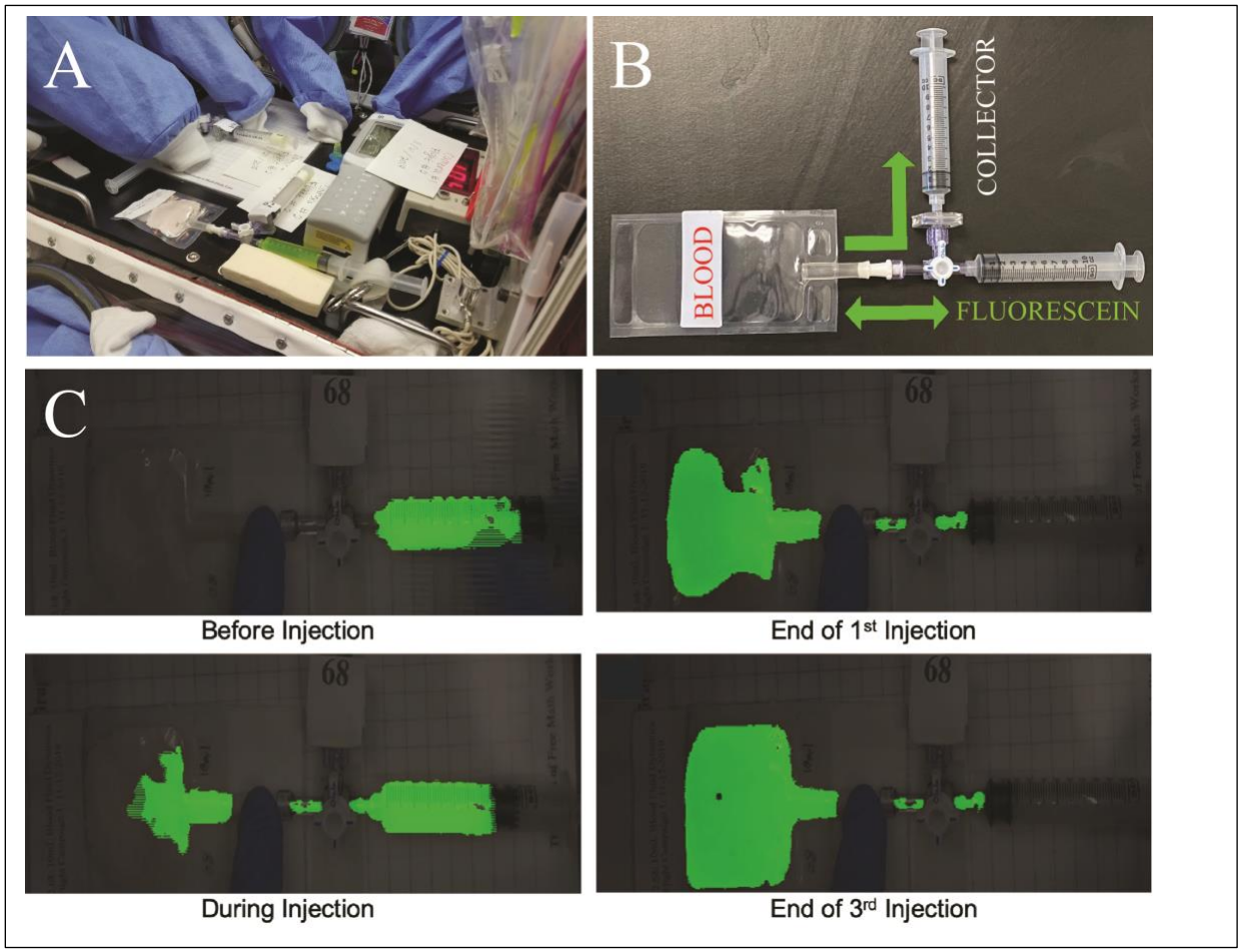

Fig. 3. Fluid dynamics during rehydration of freeze-dried RBCs were monitored at 1 *g* and 0 *g*. A) Experimental setup inside the glovebox. B) Diagram of the syringe rehydration system that was studied. C) Representative frames from MATLAB video analysis of the RBC rehydration procedures with fluorescein solution.

Fig. 4

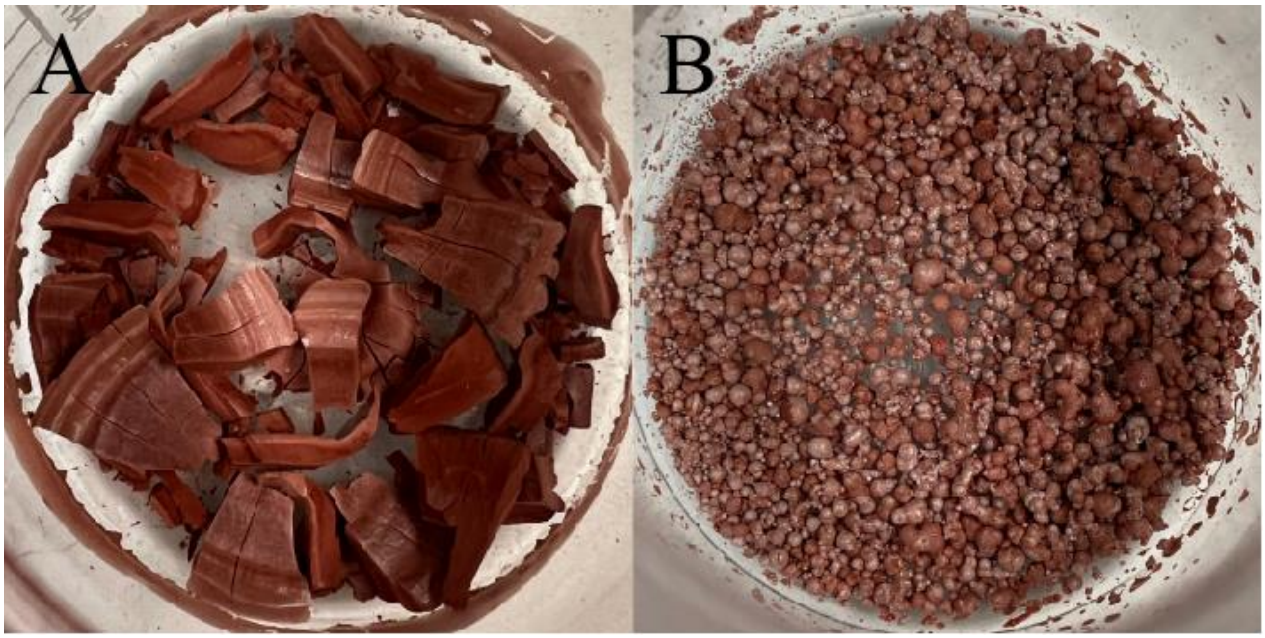

Fig. 4. Freeze-dried RBC products produced by two different freezing approaches. A) Bulk freezing before the freeze-drying step yields a lyophilized cake substance that becomes flaky when broken up to remove it from the container. B) Spray freezing before lyophilization yields spheres of varying sizes.

Fig. 5

Fig. 5. Dual-chamber rehydration bag filled with spray freeze-dried RBCs (left chamber) and PBS rehydration solution (right chamber).

Fig. 6

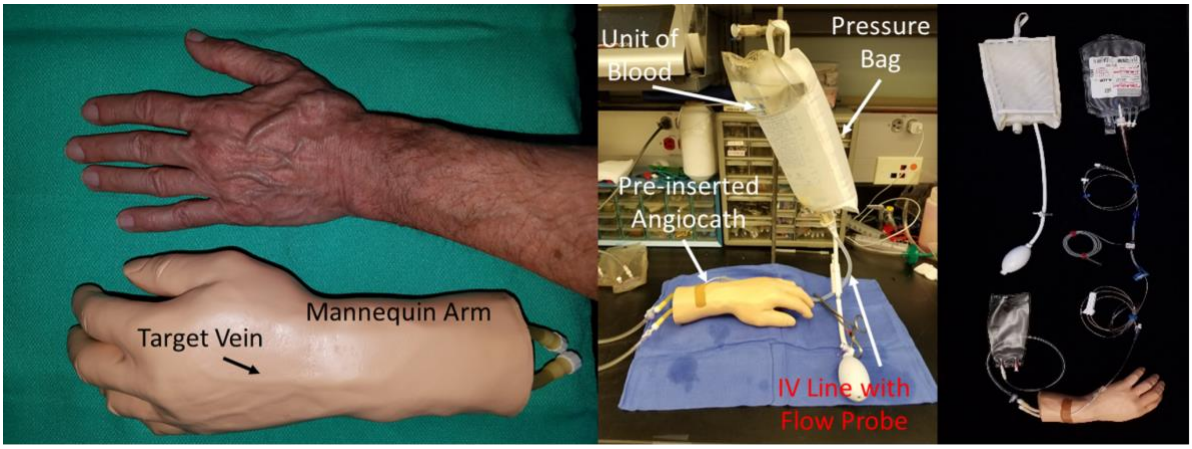

Fig. 6. Materials used for transfusion studies in a mannequin arm at different gravitational forces.

Fig. 7

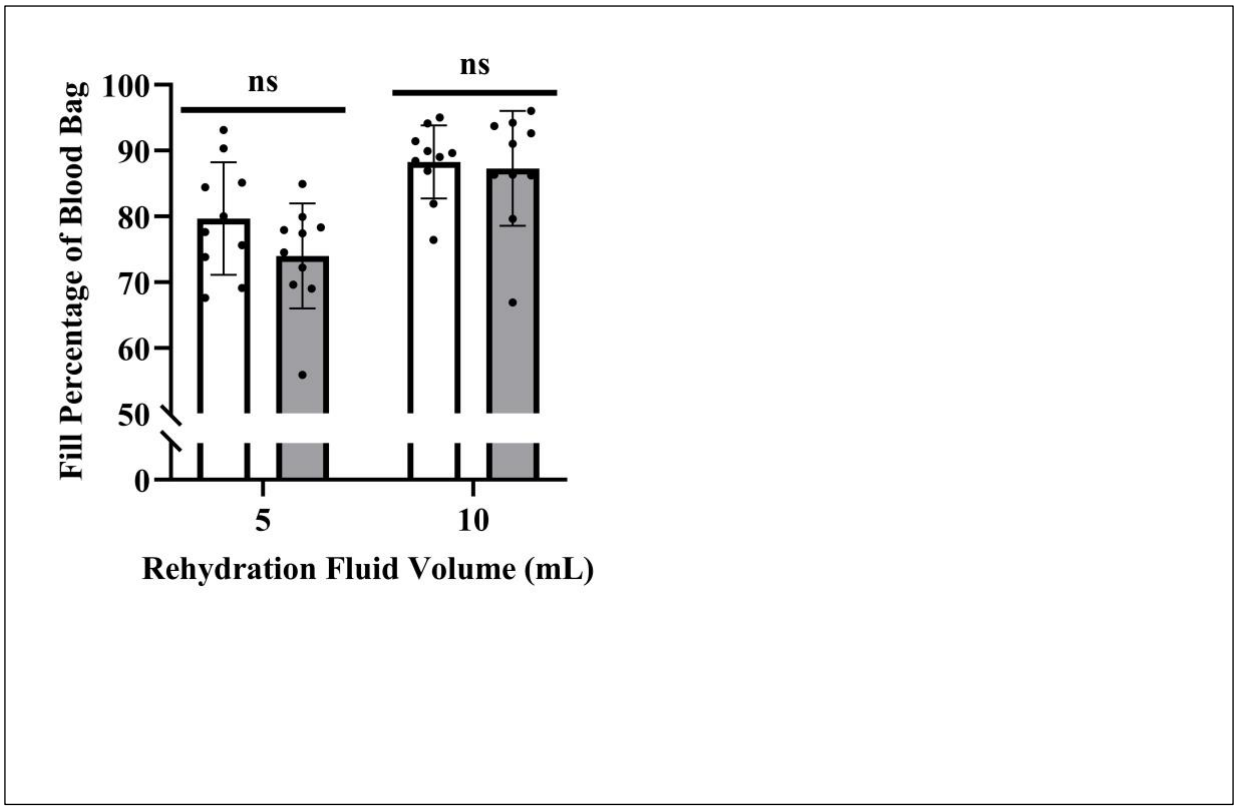

Fig. 7. Blood bag fill percentage at rehydration volumes of 5 mL and 10 mL. Fluid dynamics were analyzed at 1 *g* (white) and 0 *g* (grey). No significant difference was detected in MATLAB video analysis of the percentage of the blood bag that was filled when comparing the gravitational force experienced during the rehydration (ns = $p > 0.05$, $n = 10$).

Fig. 8

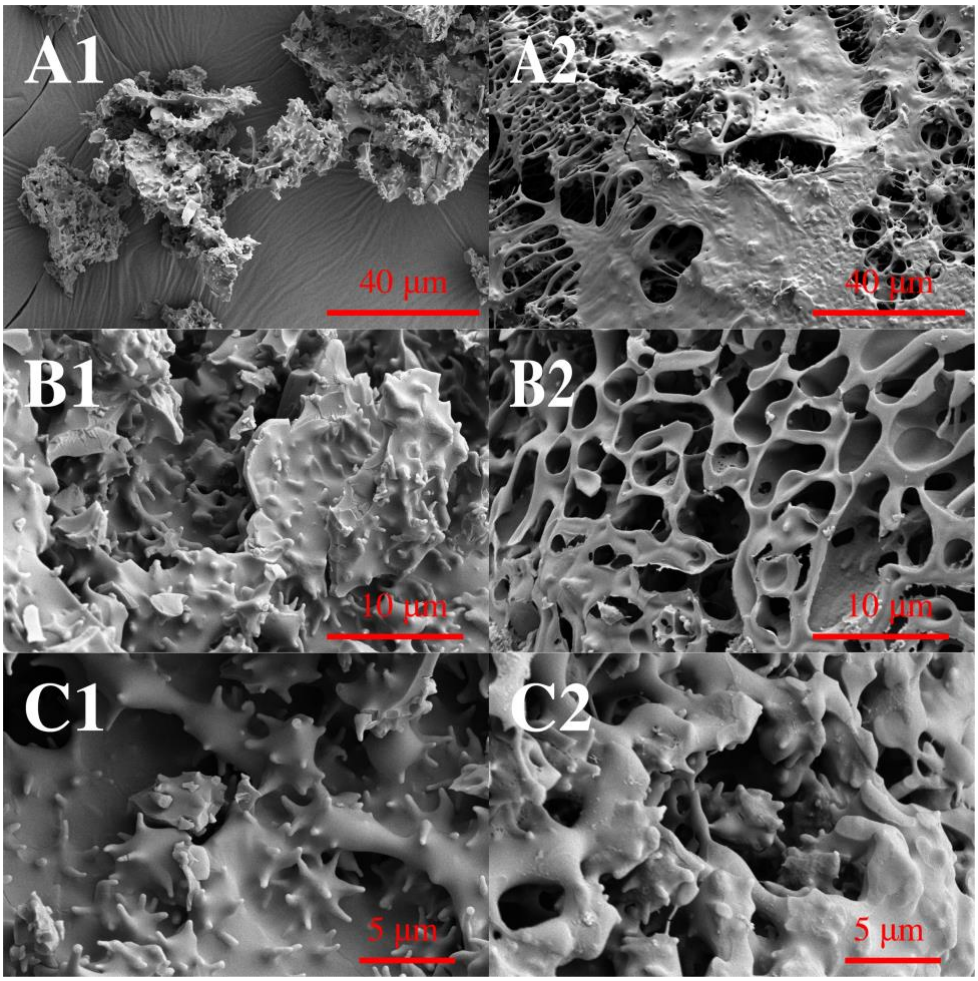

Fig. 8. Scanning electron micrographs of freeze-dried RBCs. 1) Bulk freeze-dried and 2) spray freeze-dried RBC products. Micrographs were taken at magnifications of: A) 1000X, B) 3500X, and C) 5000X.

Fig. 9

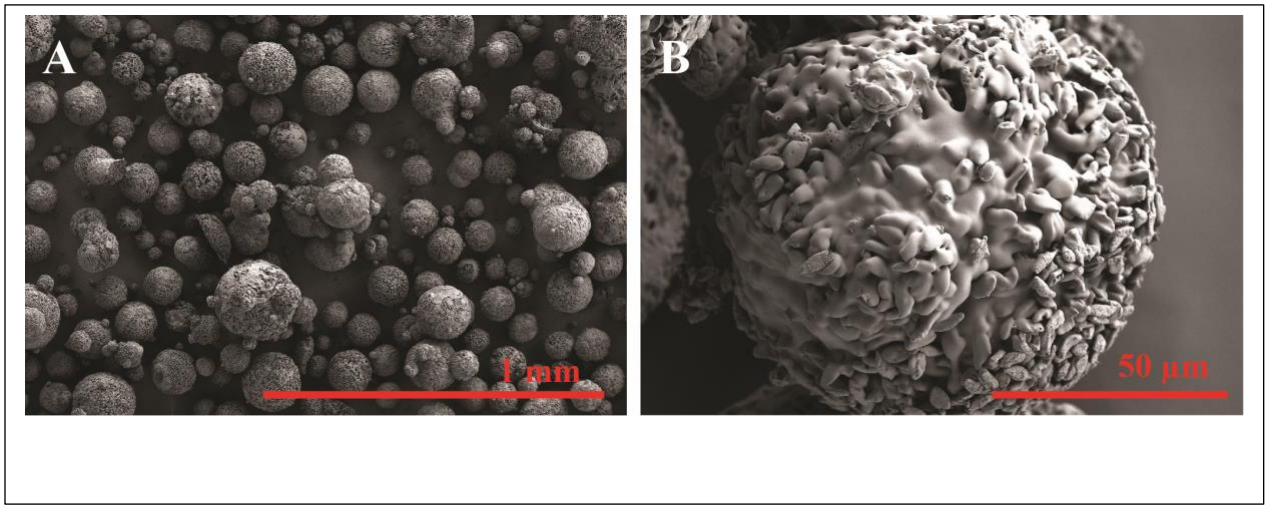

Fig. 9. Scanning electron micrograph of spray freeze-dried RBCs. Micrographs were taken at magnifications of: A) 50X and B) 1000X.

476 Fig. 10

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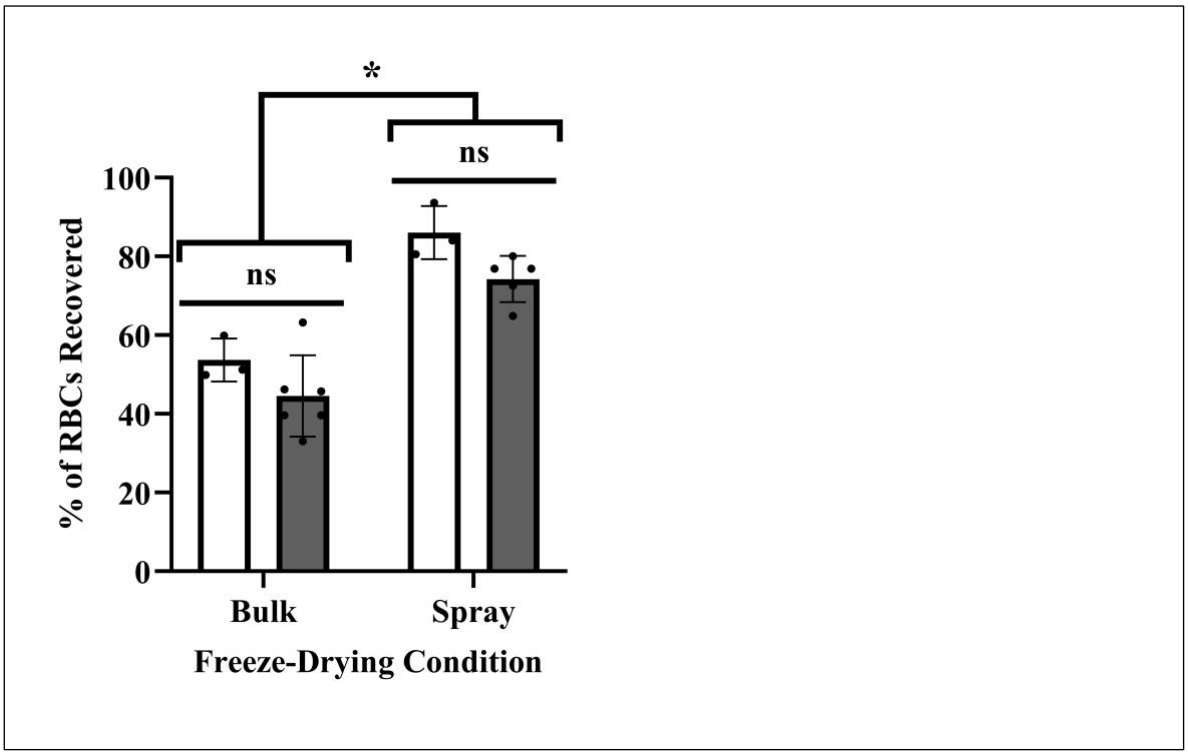

490 Fig. 10. RBC recoveries after freeze-drying, storage, and rehydration performed at 1 *g* (white) or
491 0 *g* (grey). RBCs were freeze-dried utilizing bulk freeze-drying or spray freeze-drying. *
492 Represents a significant difference compared to the corresponding bulk freeze-drying condition
493 ($p < 0.0001$, $n = 3 – 6$). A significant difference was undetectable when comparing the
494 gravitation forces occurring during the rehydration (ns = $p > 0.05$, $n = 3 – 6$).

Fig. 11

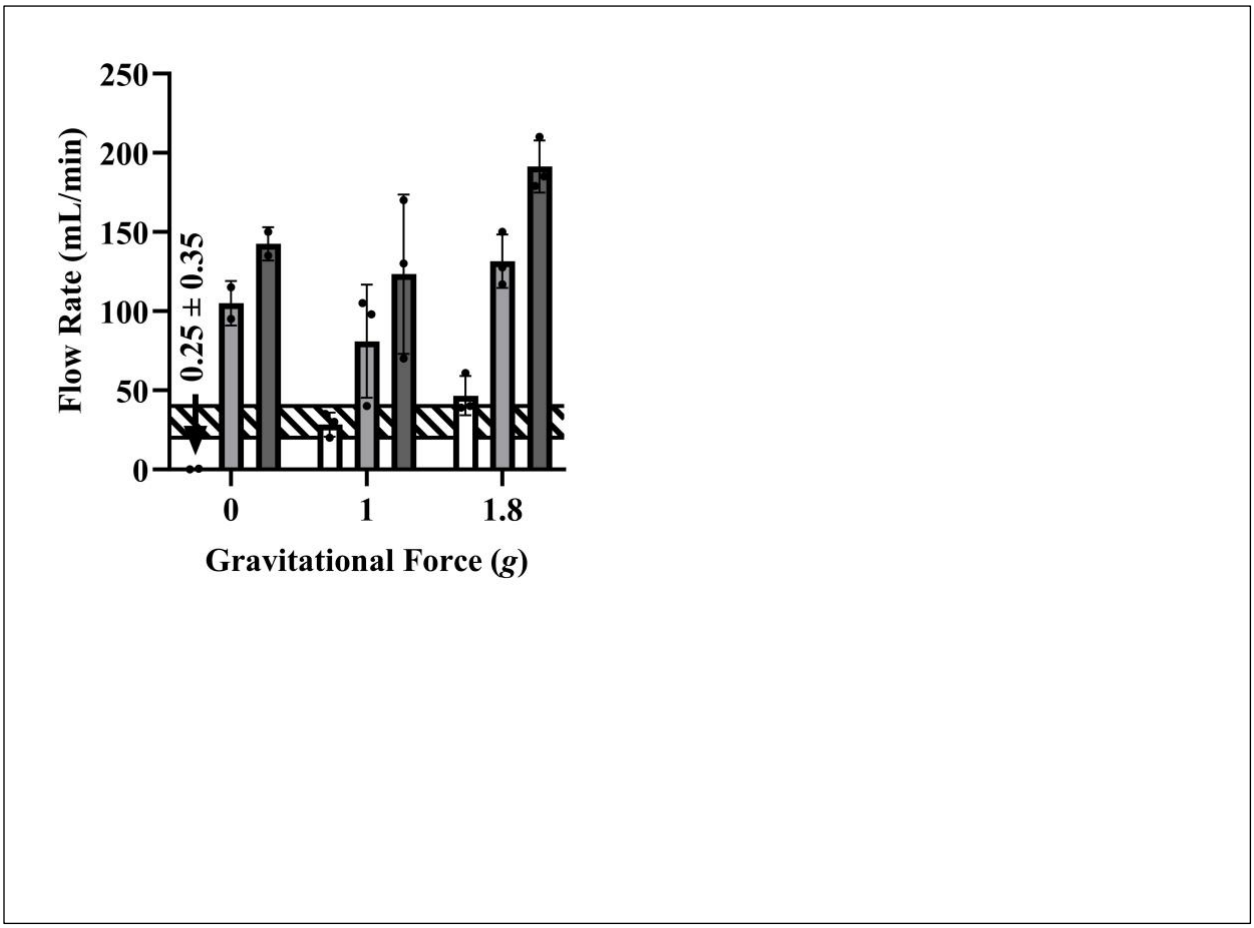

Fig. 11. Flow rates of rehydrated RBCs transfused into a mannequin arm using a SunMed Novaplus pressure infusion bag (Grand Rapids, MI). The pressure of the bag was set to 0 mmHg (white), 150 mmHg (light grey), and 300 mmHg (dark grey). The gravitational forces studied were 0 *g*, 1 *g*, and 1.8 *g*. The **striped region** represents the clinically acceptable flow ranges for rapid resuscitation.

7. Supplemental Figures

Fig. S1

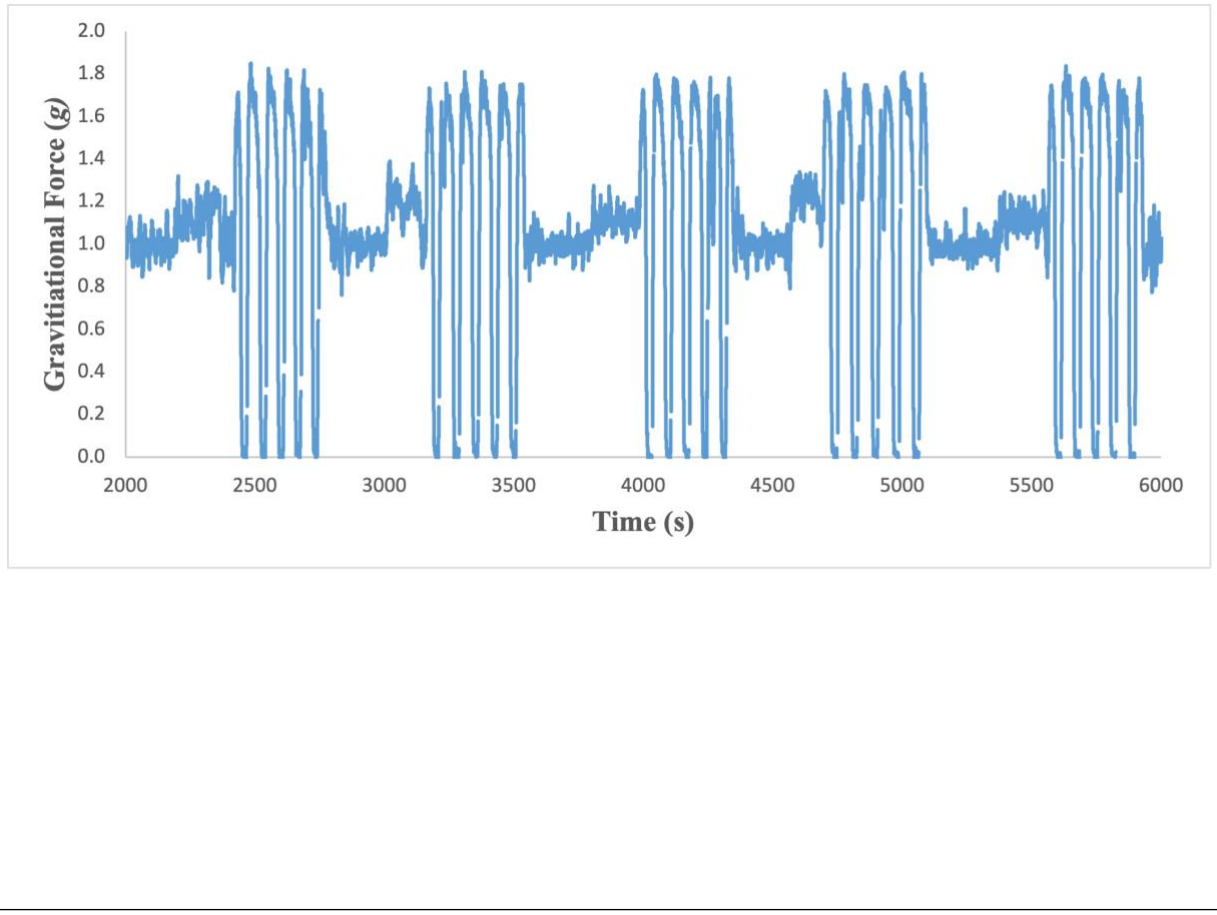

Fig. S1. Example data collected from the accelerometer during the flights that simulated reduced gravity (0 *g*) and hypergravity (1.8 *g*).

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