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Towards bedside washing of stored red blood cells: a prototype of a simple apparatus based on microscale sedimentation in normal gravity

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Abstract

Background and Objectives—Infusion of byproducts of red blood cell (RBC) storage-induced degradation as well as of the residual plasma proteins and the anticoagulant-preservative solution contained in units of stored blood serves no therapeutic purpose and may be harmful to some patients. Here we describe a prototype of a gravity-driven system for bedside washing of stored RBCs.

Materials and Methods—Stored RBCs were diluted to 10% hematocrit (Hct) with normal saline, matching the conventional washing procedure. The dilute RBC suspensions were passed through a column of coiled tubing to allow RBC sedimentation in normal gravity, thus separating them from the washing solution. Washed RBCs were collected using bifurcations located along the tubing. Washing efficiency was quantified by measuring Hct, morphology, deformability, free hemoglobin, and total free protein.

Results—The gravity-driven washing system operating at 0.5 mL/min produced washed RBCs with final Hct of 36.7 ± 3.4% (32.3 – 41.2%, n = 10) and waste Hct of 3.4 ± 0.7% (2.4 – 4.3%, n = 10), while removing 80% of free hemoglobin and 90% of total free protein. Washing improved the ability of stored RBCs to perfuse an artificial microvascular network by 20%. The efficiency of washing performed using the gravity-driven system was not significantly different than that of conventional centrifugation.

Conclusions—This proof-of-concept study demonstrates the feasibility of washing stored RBCs using a simple, disposable system with efficiency comparable to that of conventional

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Author Contributions

Shevkoplyas, Khanal, and Huynh conceived and designed the study. Khanal, Huynh, Torabian, and Xia performed the experiments and collected the data. Torabian constructed the analytical model. Shevkoplyas, Khanal, Huynh, and Vörös analyzed and interpreted the data. Shevkoplyas obtained funding and supervised the study. Shevkoplyas, Khanal, Huynh, and Vörös wrote the manuscript. All authors critically reviewed and approved the final submitted manuscript.

Conflict of Interests

The authors declare no conflict of interests.

Data and materials availability

All data and described custom-written scripts are available from the authors upon reasonable request.

centrifugation, and thus represents a significant first step towards enabling low-cost washing of stored blood at bedside.

Introduction

Most transfused red blood cells (RBCs) are collected as whole blood, leukoreduced, separated from plasma, re-suspended in an additive preservative solution and stored in a plastic bag at 2–6°C for up to six weeks. In addition to the storage medium, RBC units contain residual plasma proteins not removed during blood component separation, and leukocyte fragments remaining after leukoreduction.[1–3] During the hypothermic storage, RBC units steadily accumulate byproducts of RBC metabolism (e.g. K^+, lactate) and storage-induced degradation (e.g. microparticles, free hemoglobin).[4–8] In current medical practice all of the RBC unit's contents are infused into the patient during transfusion. However, infusion of anything other than well-preserved RBCs (e.g. metabolic wastes, microparticles, free hemoglobin, residual plasma proteins, cellular debris, and components of the storage medium) during transfusion has uncertain therapeutic purpose, and may be harmful to some patients.[9, 10] For example, free hemoglobin scavenges nitric oxide in the microvasculature, inhibiting endothelial signaling and promoting vasoconstriction and platelet activation,[8, 11, 12] and also contributes to iron overload in patients receiving frequent RBC transfusions.[13–15] The residual plasma proteins may cause severe allergic reactions in patients with IgA deficiency and those sensitized by previous transfusions.[1, 16–18]

One approach to preventing the adverse events that may be caused by toxic mediators contained in the storage medium is to wash stored RBCs in normal saline before transfusion.[19, 20] Washing of blood products is known to substantially reduce allergic transfusion reactions.[18] Washing of irradiated RBCs reduces K^+ and lactate loads, and prevents hyperkalaemia in infants and neonates undergoing cardiopulmonary bypass.[21] Data from a prospective, randomized, controlled, clinical trial suggested that washing of blood products may reduce host inflammatory responses and improve clinical outcomes in neonates and infants undergoing cardiac surgery,[22] while another randomized, clinical trial found no benefit for adult cardiac surgery patients.[23] Data from a randomized, controlled, blinded trial in critically ill beagles infected with *Staphylococcus aureus* have shown that washing of 42-day-old RBC units significantly improved clinical outcomes (including survival and multiple organ injury), while washing of 7-day-old RBCs had an opposite effect.[24] Our recent *in vitro* studies suggest that in addition to removing toxic mediators, washing of RBCs stored for prolonged periods of time could partially restore RBC deformability lost during storage.[25, 26]

At present, washing of stored RBCs can be performed using either a high-volume swing-bucket centrifuge,[25, 26] a specialized cell processor[22] or a blood salvage/autotransfusion device.[27, 28] Because of the high cost and complexity of these centrifugation-based methods, RBC washing is only performed in limited cases for which the clinical need is vital (such as for patients with known IgA deficiency). Additionally, centrifugation-based washing may subject stored RBCs to excessive mechanical stresses, causing hemolysis and increasing cell-free hemoglobin.[23, 29] In this proof of concept study we describe the

development of a prototype for a simple, inexpensive, disposable device capable of washing stored RBCs in saline without centrifugation.

Materials and Methods

Samples of stored RBCs used for washing

RBC units (CPD/AS-1, leukoreduced, storage duration 5–8 weeks) were obtained from the Gulf Coast Regional Blood Center (Houston, TX). For each experiment, a 10–20 mL blood sample was withdrawn from a unit; 5 mL was set aside for morphological and biochemical analysis, and 5–15 mL were diluted with 0.9% (normal) saline to 5–20% hematocrit (HCT), yielding a 50 mL sample which was then driven through the gravity-driven washing system at the desired rate with a programmable syringe pump (NE-1000, New Era Pump Systems, Farmingdale, NY). The system consisted of plastic tubing (19.8 m length, 1 mm internal diameter, Cole Parmer, Vernon Hills, IL) wound on a vertical cylinder, with extraction bifurcations located at various positions along the tubing.

To compare the gravity-driven washing system with conventional centrifugation-based washing, a 2 mL sample from the same unit was diluted to 10% HCT with saline and centrifuged at 1500×g for 15 minutes (Allegra X-15R, Beckman Coulter, Indianapolis, IN). The supernatant was removed for analysis, and the pellet of washed RBCs was re-suspended in saline at 30–40% HCT, matching the output HCT of the gravity-driven washing system. All sample processing procedures were performed at room temperature.

The washing quality was assessed by measuring several key parameters for the initial, diluted, and washed RBC samples, and for the washing supernatant. Initial sample refers to the unwashed RBCs withdrawn from the RBC unit. Diluted sample refers to the RBCs diluted down to 5–20% HCT with saline. Washed sample refers to RBCs washed either using our gravity-driven washing system or centrifugation.

Free hemoglobin assay

Total free hemoglobin was measured using the modified cyanmethemoglobin method following the manufacturer's instructions (D5941, Sigma-Aldrich, St. Louis, MO). The absorbance was measured at 550 nm using a plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA). Concentration of free hemoglobin was calculated using a calibration curve constructed using human hemoglobin standard (Pointe Scientific, Canton MI).

Measurement of total free protein and adenosine 5′-triphosphate

Total free protein was measured using Pierce 660 nm protein assay according to the manufacturer's instructions (Fisher Scientific, Pittsburgh, PA). The absorbance was measured using a plate reader (SpectraMax M5). Adenosine 5′-triphosphate (ATP) concentration was measured using a bioluminescent assay according to the manufacturer's instructions (FLASC, Sigma-Aldrich). The amount of light emitted was measured with a multi-mode plate reader (Victor X4, PerkinElmer, Waltham, MA).

Measurement of RBC mechanical properties

The mechanical properties of RBCs were assessed by measuring their ability to perfuse an artificial microvascular network (AMVN) as previously described in detail.[26] Briefly, HCT of RBC samples was adjusted to 40 ± 0.5% with saline. The sample was loaded into the inlets of the AMVN device, the driving pressure was set to 20 cmH_2O and the average RBC velocity was measured by analyzing sequential images of the post-capillary venules. The AMVN perfusion rate was then calculated by multiplying the average RBC velocity by the cross-section of the venule, for each AMVN network unit.

Morphological analysis

To quantify morphology, RBC sample was diluted to 1% HCT with saline, 4 μL of the diluted sample was placed on a glass slide coated with polydimethylsiloxane (PDMS) and covered with a thin slab of PDMS as a coverslip. Bright-field images of the cells were taken using an inverted microscope (IX71, Olympus America, Center Valley, PA). The cells in images were classified as discocyte, echinocyte 1, echinocyte 2, echinocyte 3, sphero-echinocyte, spherocyte or stomatocyte (673±74 cells per RBC unit), as previously described. [30]

Statistical analysis

Statistical analysis of the differences was performed with paired, two-tail Student's t-test with a significance level of 0.05. Values were expressed as mean ± standard deviation (range) unless otherwise specified.

Results

Figure 1 illustrates sedimentation of RBCs in a dilute suspension flowing through a circular tube schematically. In the beginning of the tube, RBCs are distributed uniformly throughout the lumen. The tube appears filled with blood completely, when viewed from the side. As the suspension flows further down the tube, individual RBCs sediment towards the bottom of the tube (along the vector of gravity) and the tube appears only partially filled with blood. To estimate the length of tubing, L (m), that would allow RBCs to sediment completely for a given flow rate, Q (m^3/s), we modelled RBC sedimentation – generally, a very complex, multi-scale process[31] – as a motion of a spherical particle under Poiseuille flow, as previously described:[32]

$$L = \frac{6\eta}{\pi\left(\rho_p - \rho_m\right) R^2 dg} Q$$

In this formula, $\eta = 2.0 \times 10^{-3}$ (kg/m/s) is the dynamic viscosity of the suspending medium, $d = 1.0 \times 10^{-3}$ (m) is the diameter of the tube, $\rho_p = 1125$ (kg/m^3) is the density of the particle, $\rho_m = 1070$ (kg/m^3) is the density of the suspending medium, $R = 3.5 \times 10^{-6}$ (m) is the radius of the particle, $g = 9.81$ (m/s^2) is the acceleration due to gravity. Using this equation, we estimated that at the flow rate of 2.0 mL/min (the highest flow rate we used in this study) the length of tubing needed to enable complete sedimentation of the particle was about 19 m. (Note: this model is a rough approximation meant to provide an order-of-

magnitude estimate of the length of tubing needed, rather than to predict precise movement of each sedimenting RBC.)

Figure 2 illustrates the assembled gravity-driven washing system. The system operates by passing a sample of a dilute suspension of stored RBCs through a coil of plastic tubing at a constant flow rate controlled by a syringe pump (Fig. 2 **middle panel schematic**). We implemented a two-stage separation approach, in which dilute suspension of stored RBCs first passed through the initial part of the sedimentation coil (about 15 m in length), the topmost layer of washing solution was siphoned off through a y-type bifurcation directly to waste (Fig. 2b), and the bottommost layer of washed RBCs was collected from a v-type bifurcation (Fig. 2c). The remaining blood then passed through the second part of the sedimentation coil (about 5 m in length) to allow for further sedimentation of RBCs; the washed RBCs and washing waste were separated using the second v-type bifurcation (Fig 2d, **and middle panel schematic**).

Figure 3 demonstrates the dependence of HCT of washed RBCs and waste washing solution on the flow rate of the gravity-driven washing system (Fig. 3a) and on initial HCT of the dilute RBC suspension (Fig. 3b). For stored RBCs diluted to the initial HCT of 10%, the output HCT of washed RBCs decreased with increasing flow rate from 44.1 ± 2.5% at 0.25 mL/min down to 12.2 ± 1.0% at 2.0 mL/min, and the HCT of waste washing solution increased with increasing flow rate from 1.7 ± 0.3% at 0.25 mL/min up to 8.0 ± 0.7% at 2.0 mL/min (Fig. 3a). For the gravity-driven washing system operating at 0.5 mL/min, the output HCT of washed RBCs increased from 27.7 ± 0.5% to 41.5 ± 0.8% with initial HCT increasing from 5% to 10%, and then remained relatively constant at about 42% with further increases in the initial HCT. The HCT of the waste washing solution, however, increased steadily with increasing initial HCT from 0.1% at 5% to 14.2 ± 1.5% at 20% (Fig 3b).

Figure 4 shows the dependence of the output HCT for RBCs washed using the gravity-driven washing system on the initial morphology of several different RBC units (n = 10, storage duration 5–7 weeks). The output HCT for washed RBCs across all units was 36.7 ± 3.4% (32.3 – 41.2%) and the HCT for the washing waste was 3.4 ± 0.7 (2.4 – 4.3%) at the flow rate of 0.5 mL/min. We found that units that contained a higher fraction of well-preserved RBCs produced a higher output HCT after washing ($r^2 = 0.7976$) (Fig. 4).

Table 1 shows the effect of saline washing on the composition of the suspending medium and RBC properties. Stored RBCs were diluted to 10% HCT with normal saline and passed through the gravity-driven washing system at 0.5 mL/min; the output HCT of washed RBCs was about 33% and of washing solution (waste) was about 5%. Free hemoglobin and total free protein in washed samples were both significantly lower than in the initial sample, while the AMVN perfusion rate was significantly higher ($p > 0.05$). Overall, washing reduced the concentration of free hemoglobin by more than 4-fold, and of total free protein by over 10-fold, and caused a minor (although statistically insignificant) reduction in the intracellular ATP for washed RBCs (Table 1). Further we found that the rate at which RBCs were able to perfuse an artificial microvascular network (AMVN) for washed RBCs was 22 ± 4% greater than that for the unwashed sample, signifying a substantial improvement in RBC deformability. The efficiency of washing performed using our gravity-driven washing

system or using conventional centrifugation was effectively the same for all key metrics (Table 2).

Discussion

This proof-of-concept study demonstrates the initial feasibility of a very simple, inexpensive, disposable device for washing of stored RBCs without the use of centrifugation. Our analytical model suggested that in a 20 m long, 1 mm inner diameter tube RBCs should sediment completely (Fig. 1) even at the highest flow rates used in this study, and our experimental data generally agreed with this estimate. At 0.5 mL/min, the height of RBC layer reached a plateau within 10 m of tubing length occupying 55–60% of the lumen throughout the rest of the tubing (see Supporting Information). RBCs appear to occupy more than half of the tube lumen likely due to the convective mixing at the interface between the washing solution (waste) flowing through the top part of the tube, and the layer of RBC sediment flowing through the bottom part of the tube. Mixing occurs because of the viscosity mismatch between the top clear layer depleted of RBCs (lower viscosity) and the bottom layer packed with RBCs (higher viscosity).[33]

The current prototype of the gravity-driven washing system operating at a flow rate of 0.5 mL/min produced a suspension of washed RBCs with output hematocrit of 36.7 ± 3.4% (32.3 – 41.2%, n = 10) and washing waste with a hematocrit of 3.4 ± 0.7 (2.4 – 4.3%, n = 10). All three of the key performance characteristics (throughput, product hematocrit, and product yield) must be improved before the gravity-driven washing system could compete with existing centrifugation-based systems.[28] A typical transfusion for adult recipients takes approximately 2 hours per unit of RBCs in non-urgent clinical situations, which means that our gravity-driven washing system would have to ultimately process blood diluted with saline at about 20 mL/min to enable RBC washing at bedside. To reach such a throughput, about 40 of the prototype system's sedimentation coils would have to be run concurrently. Although the relatively narrow tubing used for constructing the coils could enable packaging them without drastically increasing the footprint of the system, it is likely that the gravity-driven washing system would be most useful in situations when a significantly lower volumetric throughput is required (such as neonatal and pediatric transfusions), or where conventional centrifugation-based methods are unavailable (such as hospitals in rural areas, and other resource-limited settings). The product hematocrit could be increased by reducing the flow rate through the sedimentation coils (Fig. 3a), although such an adjustment would negatively impact the overall throughput of the system.

Our data suggest a strong dependence of product hematocrit on the initial morphological quality of stored blood – generally, RBC units that contained a larger fraction of well-preserved cells produced higher product hematocrits (Fig. 4). In this proof-of-concept study we used 6–7-week-old RBC units containing a sizable fraction of cells in advanced stages of morphological degradation. However, the average age of RBC units at the time of transfusion is less than 3 weeks.[34] Because blood stored for shorter periods of time is more likely to contain a larger fraction of well-preserved cells, we anticipate that the gravity-driven washing system would produce a higher product hematocrit for fresher RBC units than used in our study. Similarly, the use of alternative washing solutions capable of

recovering the morphology of stored RBCs[25] could also improve the washing system performance.

The washing efficiency for the gravity-driven washing system described here was comparable with that of conventional centrifugation. Washing performed by either of the two methods reduced free Hb by more than 70% and total free protein by more than 90%, while only slightly reducing intracellular ATP and yet significantly improving the ability of washed cells to perfuse an artificial microvascular network (Table 2). The reduction of free hemoglobin and total free protein after washing occurs as a consequence of diluting stored blood with a large volume of saline. In our study, we diluted the samples down to 10% hematocrit, which corresponded to washing a 350 mL, 65% hematocrit RBC unit with 2000 mL of saline, and the maximum possible reduction in the concentration of total free protein of about 94%. We chose such a dilution to emulate the dilution typically occurring during the conventional, centrifugation-based washing procedure. We found that the concentration of total free protein was reduced by about 97% with centrifugation-based washing, and by about 92% with gravity-driven washing (Table 2), which compares well with what should be expected based on the dilution ratio. Such a performance (by either approach), however, may not be sufficient for hypersensitive recipients, such as IgA-deficient individuals. For such patients a reduction in total free protein of 99% or more is highly desired, and double washing is recommended to ensure the nearly complete removal of residual proteins.[35]

In this study, saline washing increased the fraction of well-preserved cells (discocyte, echinocyte 1) from 22.6 ± 15.6% in the initial to 47.6 ± 1.4% in the washed sample, and reduced the fraction of poorly-preserved cells (echinocyte 2, echinocyte 3) from 71.5 ± 14.6% in the initial to 46.5 ± 0.6% in the washed sample, while leaving the fraction of irreversibly damaged cells (sphero-echinocytes, spherocytes and stomatocytes) effectively unchanged (5.9 ± 1.1% initially, 5.9 ± 1.1% washed). Our previous *in vitro* studies suggest that the change in RBC shape from discocyte to sphero-echinocyte may contribute substantially to the loss of RBC deformability observed during hypothermic storage.[36] The significant improvement in the ability of stored RBCs to perfuse an artificial microvascular network (AMVN) (Table 1 and 2) can be explained, therefore, by normalization of RBC morphology after washing. Interestingly, both gravity-driven and centrifugation-based washing produced a similar increase in the AMVN perfusion rate (Table 2), suggesting that recovery of RBC deformability after washing we reported previously[25, 26] and observed in this study is independent of the mechanical stress experienced by stored RBCs during centrifugation.

The prototype of the gravity-driven washing system presented here represents an important first step towards the development of a simple, disposable, low-cost device for washing of stored RBCs without the use of centrifugation. Such a device would be fundamentally more portable than current centrifugation-based methods, and could perform washing either as an independent closed system, or in line with existing infusion equipment at bedside. The disposable nature of the gravity-driven washing device would eliminate the need for the substantial capital investment into centrifugation-based cell washers, and thus could significantly reduce the cost of washing, particularly for hospitals that perform washing infrequently. Importantly, a disposable washing device would be inherently scalable, and

thus it could effectively enable washing of many RBC units simultaneously, which is very costly to accomplish using current centrifugation-based cell processors. Finally, simplicity of the device could make it a particularly attractive solution for resource-limited settings (e.g. developing countries lacking reliable infrastructure that is required for operating and maintaining centrifugation-based cell washers).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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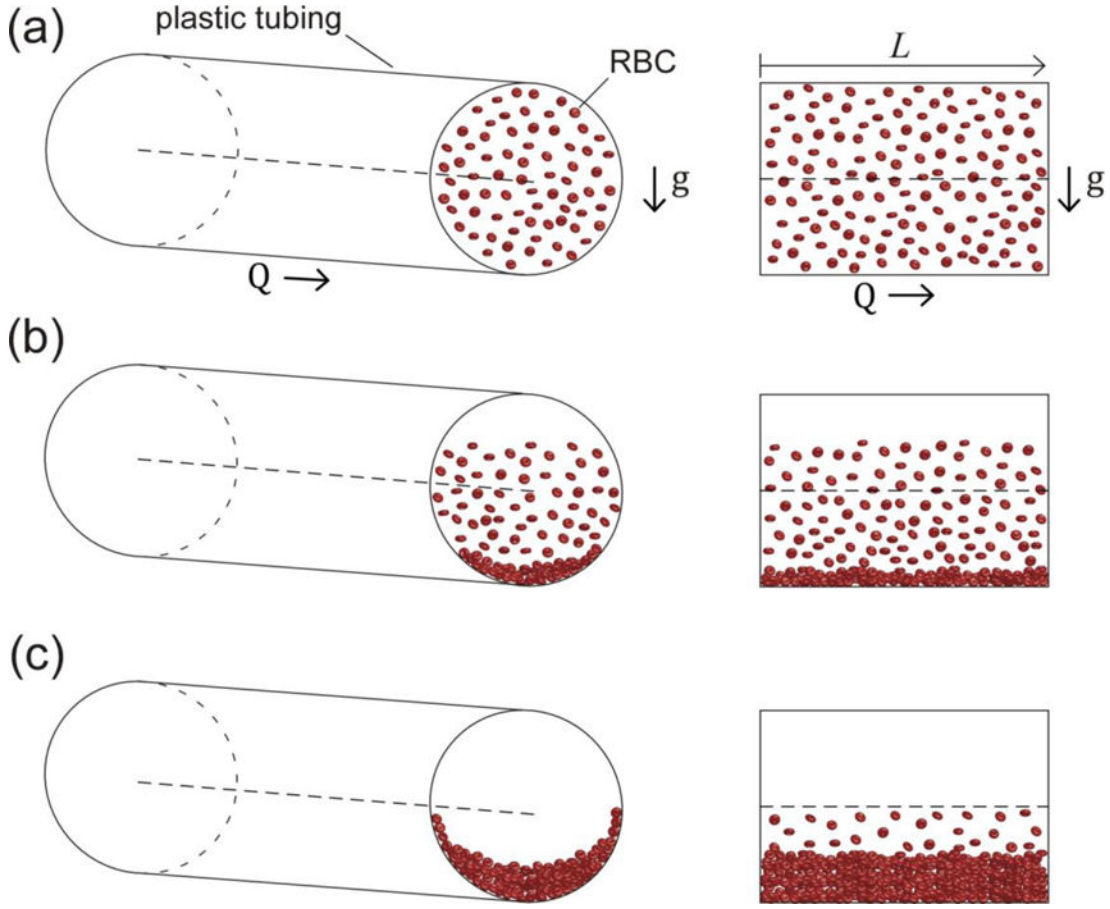

Figure 1.

RBC sedimentation in a dilute suspension flowing through a circular tube. Insets on the right show the view of the tube from the side. Arrows indicate flow direction, Q, the direction of gravity, g, and the direction of increasing length of the tube, L. (**a**) Initially, RBCs are distributed uniformly throughout the lumen. (**b**) As the RBC suspension is carried by the flow further down the tube, individual RBCs gradually sediment towards the bottom half of the tube. (**c**) Further down the tube, RBCs sediment completely towards the walls of the bottom half of the tube. When viewed from the side, the tube appears filled with blood to about half.

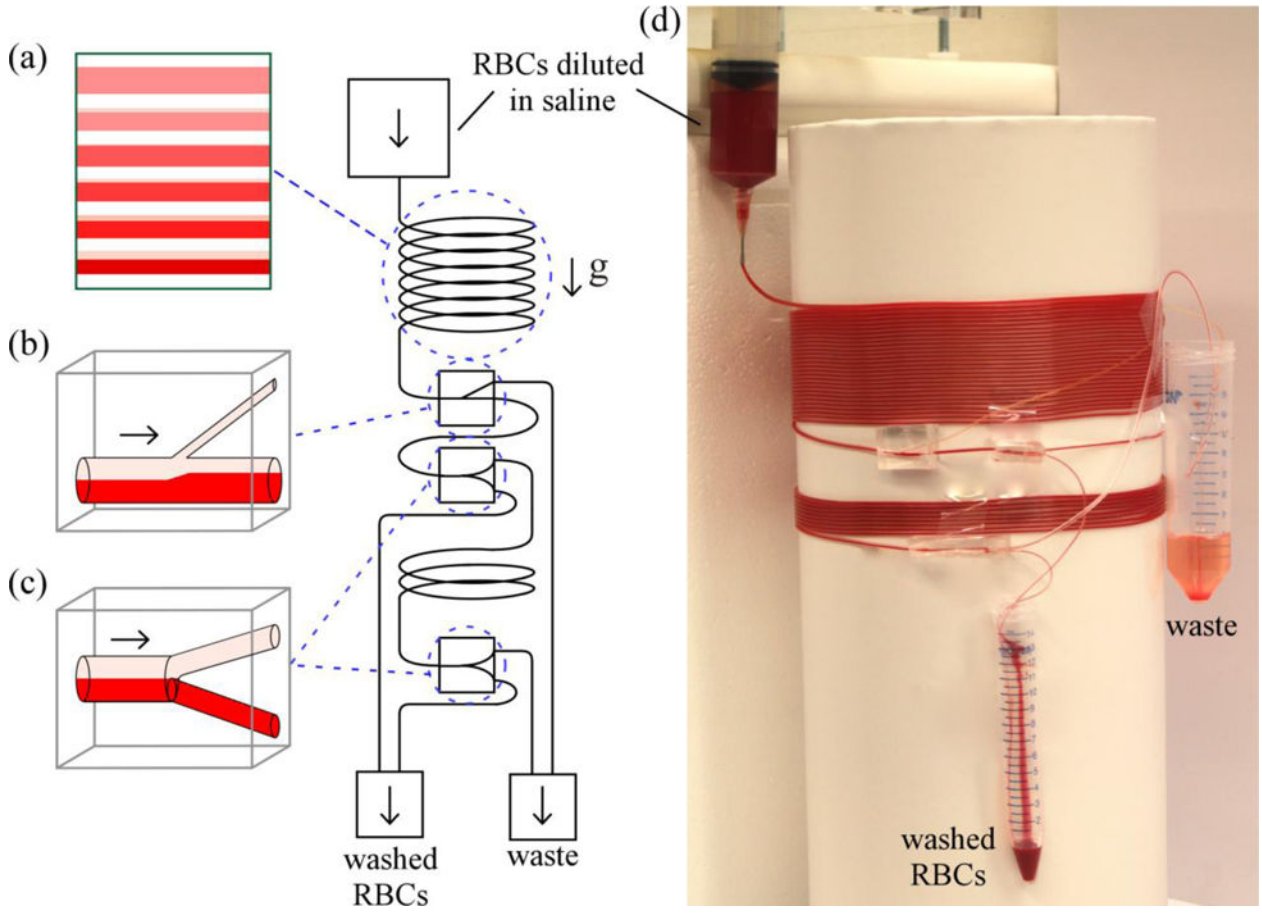

Figure 2.

The gravity-driven washing system. (**a**) Schematic illustration of the side view of the coiled tubing depicting progressive reduction of the fraction of the tubing lumen occupied by RBCs due to sedimentation (pink color represents a dilute suspension of RBCs in saline, and red color indicates an increase in concentration of the RBC layer as the RBCs sediment and pack at the bottom of the tubing). (**b**) Geometrical configuration of the y-type bifurcation. (**c**) Geometrical configuration of the v-type bifurcation. (**d**) A photograph of the assembled gravity-driven washing system, consisting of a two-part sedimentation coil and three bifurcations for extracting the washed RBCs and washing waste. The flow of sample through the system is driven by a pre-programmed syringe pump (not shown).

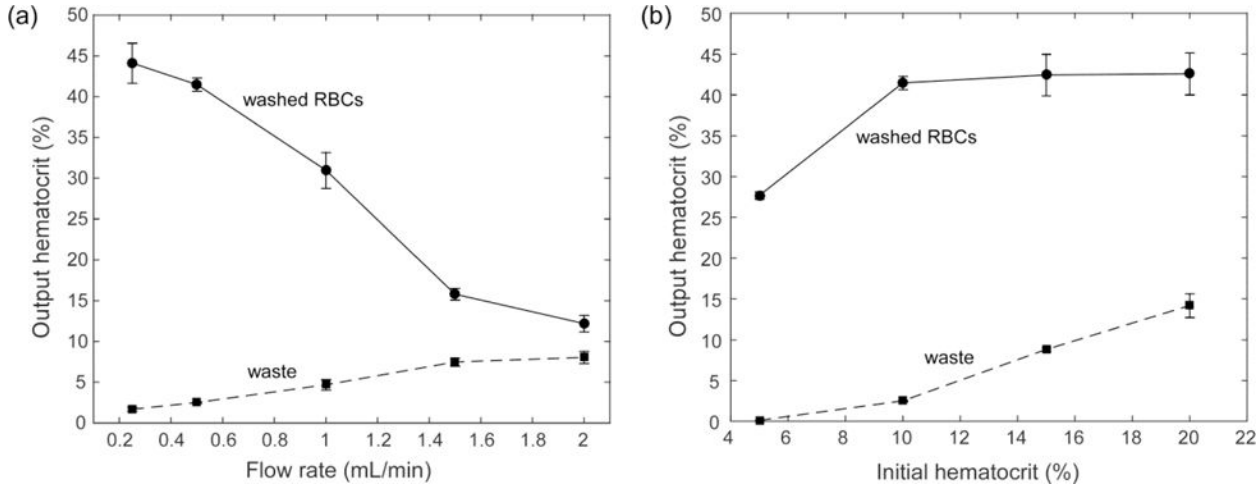

Figure 3.

The dependence of the output hematocrit for washed RBCs and washing waste on flow rate and initial hematocrit. (**a**) Stored RBCs diluted with normal saline down to 10% hematocrit passed through the gravity driven washing system at flow rates varying from 0.25 to 2.0 mL/min. The resulting output hematocrit of washed RBCs and the washing waste is reported as a function of the flow rate. Values shown are means ± SD (n = 3). (**b**) Stored RBCs were diluted with normal saline down to 5, 10, 15 or 20% hematocrit and passed through the gravity-driven washing system at a flow rate of 0.5 mL/min. The resulting output hematocrit of washed RBCs and the washing waste is reported as a function of the hematocrit of the initial sample. Values shown are means ± SD (n = 3).

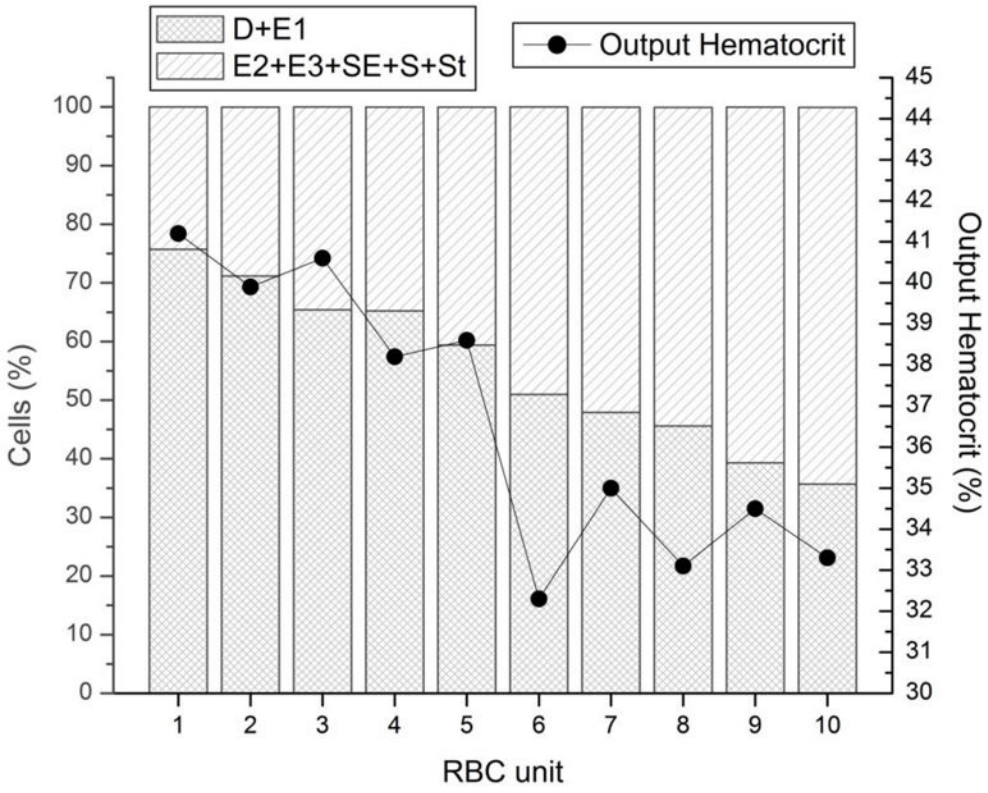

Figure 4.

The dependence of the output hematocrit for washed RBCs on morphology of stored RBCs (at the flow rate of 0.5 mL/min). Based on their shape, RBCs were classified as belonging to either of two categories: well-preserved (D, discocyte; E1, echinocyte 1) or poorly-preserved (E2, echinocyte 2; E3, echinocyte 3; SE, sphero-echinocyte; S, spherocyte; St, stomatocyte). Left Y-axis shows the percent of cells belonging to either of the two categories, and right Y-axis shows the output hematocrit for washed RBCs from each of the RBC units tested (n = 10).

Table 1

The effect of saline washing using the gravity-driven system on the properties of stored RBCs.

	Free hemoglobin (mg/mL)	Total free protein (mg/mL)	Intracellular ATP (μmol/gHb)	AMVN perfusion rate (nL/s)
Initial	1.59 ± 0.06 *	16.9 ± 0.36 *	3.74 ± 0.44	5.78 ± 0.48 *
Diluted	0.33 ± 0.01	1.63 ± 0.09	3.44 ± 0.38	–
Washed	0.37 ± 0.04 *	1.66 ± 0.07 *	3.67 ± 0.19	7.11 ± 0.67 *
Washing Solution	0.34 ± 0.01	1.63 ± 0.09	–	–

Values are mean ± standard deviation (n = 3);

* indicates statistical significance ($p > 0.05$).

Table 2

Comparison of the effect of saline washing performed the gravity-driven washing system and conventional centrifugation.

	Free hemoglobin	Total free protein	Intracellular ATP	AMVN perfusion rate
Gravity-based washing	−79.7 ± 2.7 %	−92.4 ± 0.04 %	−1.42 ± 6.40 %	+22.9 ± 5.70 %
Centrifugation-based washing	−73.0 ± 3.1 %	−97.2 ± 1.00 %	−4.63 ± 1.43 %	+19.52 ± 2.87 %

Positive values indicate increase, and negative values indicate decrease with respect to the initial sample. Values shown are mean ± standard deviation (n = 3).