Hemodilution Increases the Susceptibility of Red Blood Cells to Mechanical Shear Stress During *In Vitro* Hemolysis Testing

Christian R. Sargent,*† Ina Laura Perkins,†‡ Venkateswarlu Kanamarlapudi,† Christopher Moriarty,* and Sabrina Ali*

The American Society for Testing and Materials (ASTM) F1841 standard for the assessment of hemolysis in blood pumps recommends using phosphate-buffered saline (PBS) for hemodilution to standardize hematocrit (HCT). However, PBS increases red blood cell mechanical fragility and hemolysis. Herein, we investigated diluents and dilutions during *in vitro* testing to reduce hemodilution bias when assessing hemolysis. Bovine blood was diluted with either PBS or PBS+4/6 g% bovine serum albumin (BSA) to a 70/90% blood dilution, or to an HCT of 30%±2%, and pumped with the CentriMag or RotaFlow under hemodynamic conditions. Separately, bovine and human blood were subjected to ventricular assist device–like shear stress using a vortex. Plasma-free hemoglobin levels, normalized milligram index of hemolysis (mgNIH), and protein concentrations were analyzed. Hemolysis depended on the diluent and final blood concentration. Seventy percent of blood diluted with PBS alone caused significantly greater hemolysis than PBS+4/6 g% BSA. However, at 90% blood, PBS+4/6 g% BSA caused significantly greater hemolysis than PBS alone. Hence, a positive correlation between mgNIH and hemodilution was observed with PBS and a negative correlation with PBS+4g% BSA. PBS alone significantly reduced the total protein concentration. Hemodilution with BSA maintains protein concentration within a physiologic range and reduces bias during hemolysis testing at high blood dilutions. Thus, American Society for Testing and Materials standards could consider including BSA as a diluent, when and as required: where large dilution is required (<83%) use PBS+4 g% BSA, otherwise use PBS alone. *ASAIO Journal* 2021; 67:632–641.

Key Words: bovine, hemolysis, ventricular assist device, CentriMag, hemodilution, RotaFlow

INTRODUCTION

The evaluation of implantable medical devices is essential to investigate blood damage generated by blood pumps such as ventricular assist devices (VADs).[1,2] *In vitro* hemolysis testing is conducted in mock circulatory loops to reliably measure and characterize red blood cell (RBC) damage in relation to a baseline control, such as the widely used CentriMag extracorporeal blood pump (Thoratec).[3] Hemolysis is defined as the damage of RBCs, measured by the rate of free hemoglobin released into the surrounding plasma in response to shear stress.[4,5] The levels of plasma free hemoglobin (pfHb) can be measured over a given time interval to calculate the normalized milligram index of hemolysis (mgNIH).[1,4,6] Hemolysis testing has become an important tool for assessing the hemocompatibility, safety, and efficacy of medical devices.[2,7,8] When conducting *in vitro* hemolysis testing, the most recent American Society for Testing and Materials (ASTM) F1841-19 standard specifies that "the blood subjected to the test should have the hematocrit value adjusted by hemodilution with phosphate-buffered saline (PBS)."[1] Nevertheless, hemodilution of RBCs with PBS has been shown to significantly increase the susceptibility of RBCs to shear stress compared with RBCs diluted with serum and plasma.[9] Hemodilution with PBS alone may introduce an unwanted bias, resulting in increased hemolysis and a skewed mgNIH. This bias may be due to the reduction of protein concentration and oncotic pressure.[10] The protective effect of plasma may be attributed to the attachment of negatively charged plasma proteins, such as albumin, to the RBC surface, thus, attracting sodium ions. The resulting osmotically active layer may protect the RBCs from excess water accumulation.[11] The addition of PBS with physiologic levels of albumin (3.5–5 g/dL) has been shown to increase the mechanical resistance of washed RBCs in various models of mechanical shear, similar to the resistance shown by RBCs in plasma.[9,12–14] However, the ASTM does not raise hemodilution as an area for concern. Using PBS for hemodilution is so well established that a multilaboratory study of flow-induced hemolysis using the FDA Benchmark Nozzle Model followed this practice.[5] Therefore, in this study, we evaluated the effects of hemodilution on the susceptibility of RBCs to mechanical shear stress during *in vitro* hemolysis testing. Using two extracorporeal pumps commonly used for *in vitro* VAD hemolysis research,[3,15–17] we diluted bovine blood to various concentrations in PBS, with and without bovine serum albumin (BSA). The extracorporeal VADs used were the CentriMag and the RotaFlow, which were selected due to their demonstrated excellent safety profile for hemolysis over long-time periods in clinical settings. Thus, the hemolysis profiles obtained in this *in vitro* study could be considered clinically acceptable hemolysis levels.

MATERIALS AND METHODS

Bovine Blood Preparation

Bovine blood was collected from an abattoir and prepared as described previously.[18] Briefly, whole bovine blood was collected into 14% citrate phosphate dextrose adenine-1

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anticoagulant solution, antibiotics (50 mg/L gentamycin), and antimycotics (10 ml/dL) (Sigma Aldrich, Poole, United Kingdom). To cover the range of dilutions expected during *in vitro* hemolysis testing, the blood was diluted to a high dilution (70% blood) or a low dilution (90% blood). The blood was also diluted to achieve a hematocrit (HCT) of 30% ± 2%. Blood was diluted with PBS (Cat# 10010-015, Thermo Fisher Scientific, Basingstoke, United Kingdom) or PBS with 4 g% or 6 g% BSA (Cat# A7030, Sigma Aldrich, Poole, United Kingdom) and transferred into the test circuit within 2–3 hours after sample collection.[1]

Human Blood Preparation

Human whole blood was collected from healthy volunteers into Vacuette blood collection tubes containing citrate phosphate dextrose adenine (Greiner Bio-One, Wemmel, Belgium). Use of human blood was approved by the Wales Research Ethics Committee 6 (study number 13/WA/0190), and all donors gave informed written consent.

Device Operation and Specifications

The CentriMag Centrifugal Pump (Abbott Laboratories, Abbott Park, IL) and RotaFlow (Maquet, Rastatt, Germany) extracorporeal VADs were tested using an *in vitro* circuit under constant hemodynamic conditions for 6 hours, according to the ASTM standard[1] as previously described.[3,19] In brief, this includes a differential pressure of 100 ± 3 mm Hg and a flow of 5 ± 0.25 L/min.

Cell Counts

HCT measurements were determined in triplicate using the automated hematology analyzer Abacus Junior Vet5 (Diatron, Budapest, Hungary). Automated HCT measurements were validated using a manual comparison. The blood was drawn into microcapillary tubes (Hawksley and Sons Ltd., Lancing, United Kingdom) and centrifuged at 4,700 × g for 7 minutes. The HCT values were measured using a microcapillary HCT reader (Hawksley and Sons Ltd.).

In vitro Model of VAD-Like Shear Stress

To mimic VAD-like shear stress conditions experienced in continuous flow devices, diluted blood from bovine and human donors was exposed to a continuous cycle on a vortex (Vortex genie-2, Scientific Industries, Bohemia, NY). Aliquots of 1 ml human and bovine blood were placed in 1.5 ml Eppendorf tubes (STARLAB) and maintained at 2,400 rpm at room temperature, with a shear stress of ~175 dyne/cm^2, as previously described.[20] Blood was subjected to 360 or 20 minutes of continuous shear in bovine and human blood, respectively, to obtain a final pfHb level of 0.5 g/L, regarded as clinical hemolysis.[21] Human blood is more susceptible to shear stress than bovine blood, therefore, the time taken to release 0.5 g/L pfHb was less.[18]

Hemolysis Assay

Levels of pfHb were calculated using the Harboe direct spectrophotometric method.[22] Three 1 ml whole blood samples were collected hourly and centrifuged at 4,700 × g for 7 minutes. The plasma (100 µL) was transferred into a deep well 96-well plate (StarLabs, Milton Keynes, United Kingdom) and diluted with 1 ml of 0.1% Na_2CO_3 solution (Sigma-Aldrich, Poole, United Kingdom). The diluted plasma (170 µL) was transferred into a 96-well flat-bottom plate (ELISA plate, Greiner Bio-One, Stonehouse, United Kingdom). The absorbance was measured at the following wavelengths: 380, 415, and 450 nm (POLARstar Omega, BMG LABTECH Ltd., Aylesbury, United Kingdom). Levels of pfHb were calculated using Equation 1, as previously described.[23]

$$\text{pfHb}\left(\frac{\text{g}}{\text{L}}\right) = \left(167.2 \times A_{415} - 83.6 \times A_{380} - 83.6 \times A_{450}\right) \times \left(\frac{1}{1000}\right) \times \left(1 / \frac{\text{Vol}_{\text{plasma}}}{\text{Vol}_{Na_2CO_3}}\right) \quad (1)$$

The normalized milligram index of hemolysis (mgNIH) was calculated as described (Equation 2).[1]

$$\text{mgNIH}\left(\frac{\text{mg}}{100\ \text{L}}\right) = \Delta\text{pfHb} \times V \times \frac{100 - \text{HCT}}{100} \times \frac{100}{Q \times T} \quad (2)$$

where

mgNIH = normalized milligram index of hemolysis (mg/100L)

ΔpfHb = increase of plasma free hemoglobin concentration (g/L) over the sampling time interval

V = Circuit volume (L)

Q = Flow rate (L/min)

HCT = hematocrit (%)

T = Sampling time (min)

Previously, we have identified an acceptable limit of hemolysis using the CentriMag device.[3,24] Therefore, a mgNIH threshold was set to ≤2.0 mg/100 L. Background pfHb levels in the blood before the start of a test were corrected for, and the subsequent increase in pfHb levels was normalized using the equation for mgNIH. As such, only the released pfHb between 2 timepoints (0–6 h) were analyzed.

Protein Concentration Measurement

Total protein concentration in plasma was determined using the bicinchoninic acid (Sigma-Aldrich, Poole, United Kingdom) assay kit as per the manufacturer's instructions.

Statistical Analysis

The mean and standard deviation (SD) were calculated for all parameters and time points. A set of repeated measures analysis of variance (one-way or two-way analysis of variance) was used to analyze mgNIH, protein concentration, or HCT measurements. These data measurements were obtained from a normally distributed population. Tukey's post hoc tests were used to perform multiple comparisons. Paired t-tests were applied to compare differences in pfHb release. A Kruskal-Wallis was used to analyze pfHb release over time from the CentriMag and RotaFlow. Paired data showing standardized pfHb release in bovine and human blood were analyzed using Wilcoxon signed-rank test. Data were standardized relative to PBS to evaluate alterations in hemolysis while minimizing donor variability. All statistical data analyses were performed using the GraphPad Prism 8 software (v.8.1.1, San Diego, CA).

RESULTS

Donor Characteristics

The average HCT for all bovine donors used was 39.57% ± 3.80% ($n = 26$). Following comparative analysis, there was no significant difference in HCT between sex, age, and breed of bovine donors. Donor characteristics can be found in **Table 1**.

Hemolysis

Normalized Milligram Index of Hemolysis. Bovine blood was diluted to 70% and 90% with PBS alone or PBS + 4/6 g% BSA, for hemolysis testing using the RotaFlow and CentriMag devices. The mgNIH was calculated after 360 minutes of testing.

Hemodilution With PBS. Blood diluted with PBS showed significantly higher hemolysis in the CentriMag at a 70% dilution compared with blood diluted to 90% (5.53 ± 1.67 and 1.77 ± 0.55 mgNIH, respectively, $P = 0.0079$) (**Figure 1**). However, dilution with PBS did not significantly affect hemolysis in the RotaFlow.

Hemodilution With PBS + 4 g% BSA. Blood diluted with PBS + 4 g% BSA showed lower hemolysis in the CentriMag at a 70% dilution compared with blood diluted to 90% (0.97 ± 0.31 and 3.09 ± 0.82 mgNIH, respectively) (**Figure 1**). The same pattern was observed in the RotaFlow (1.23 ± 0.40 and 3.27 ± 1.90 mgNIH, respectively) (**Figure 1**).

Hemodilution With PBS + 6 g% BSA. Blood diluted with PBS + 6 g% BSA showed lower hemolysis in the CentriMag at a 70% dilution compared with blood diluted to 90% (1.97 ± 0.64 and 3.03 ± 1.50 mg/NIH, respectively) (**Figure 1**). The same pattern was observed in the RotaFlow (1.07 ± 0.31 and 2.3 ± 0.44 mgNIH, respectively) (**Figure 1**).

Hemolysis Between Devices

At a 70% hemodilution, the CentriMag caused significantly higher hemolysis with PBS alone than PBS + 4 g% BSA and PBS + 6 g% BSA ($P = 0.0007$; $P = 0.0141$, respectively). Hemolysis levels in the RotaFlow were not affected by PBS ± 4/6 g% BSA at a 70% dilution (**Figure 1**). However, blood diluted to 90% with PBS + 4 g% and PBS + 6 g% BSA increased hemolysis compared with PBS alone, in both devices. Compared with the CentriMag at a 70% dilution with PBS, hemolysis was significantly reduced in the RotaFlow at a 70% dilution with PBS, PBS + 4 g% BSA and PBS + 6 g% BSA ($P = 0.0006$; $P = 0.0016$, $P = 0.0010$, respectively). Additionally, compared with the CentriMag at a 70% dilution with PBS, hemolysis was significantly reduced in the RotaFlow at a 90% dilution with PBS and PBS + 6 g% BSA ($P = 0.0065$; $P = 0.0353$) (**Figure 1**).

Table 1. Comparison of Bovine Donor Characteristics Used for *In Vitro* Hemolysis Testing

Characteristic	Type	Number
Sex	M	18
	F	8
Age (y)	1	1
	2	21
	3	4
Breed	Aberdeen Angus	4
	Blonde D'aquitaine	3
	British Blue	3
	British Friesian	2
	Hereford	1
	Holstein Friesian	3
	Limousin	4
	Salers	1
	Short Horn	1
	Simmental	2
	Swedish Red Cross	1
	Welsh Black	1

Levels of pfHb Release

To analyze the effect of blood dilution on hemolysis, pfHb levels were compared during *in vitro* device testing. Total pfHb levels were increased over time; however, there were no significant differences at each time point between diluents for each dilution or device.

In the CentriMag device, a 70% hemodilution with PBS alone caused a significant increase in pfHb levels at 360 minutes compared with 0 minutes ($P = 0.0402$) (**Figure 2A**). However, the time-dependent increase in pfHb levels was reduced greatly by adding 4 g% or 6 g% BSA to the diluent PBS. PBS + 4 g% BSA caused the lowest pfHb release.

In the CentriMag device, a 90% hemodilution with 4 g% BSA caused a significant increase in pfHb levels at 360 minutes compared with 0 minutes ($P = 0.0062$) (**Figure 2B**). Hemodilution with PBS resulted in the lowest pfHb release at a 90% dilution.

In the RotaFlow device, all diluents at a 70% hemodilution caused a similar increase in pfHb levels. However, PBS is the only diluent to cause a significant increase at 360 minutes compared with 0 minutes ($P = 0.0260$) (**Figure 2C**).

In the RotaFlow device, all diluents at a 90% hemodilution caused a similar increase in pfHb levels. There were no significant differences observed in pfHb levels between each time point or diluent (**Figure 2D**).

Hematocrit Measurements

The final HCTs used in 90% hemodilution were significantly higher than the HCT used in 70% diluted bovine blood ($P < 0.0001$). However, there were no significant differences between HCT values within each condition (**Figure 3**). A low HCT in the CentriMag diluted 70% with PBS resulted in a high mgNIH (5.53 ± 1.67 mg/100 L). However, the addition of PBS + 4/6 g% BSA resulted in a lowered mgNIH (0.97 ± 0.31 mg/100 L and 1.97 ± 0.64 mg/100 L, respectively). Therefore, HCT is not the reason for the discrepancies between mgNIH results.

Protein Concentration

The total protein concentration in undiluted bovine blood ranged from 5.2 to 8.4 g/dL ($n = 16$), with a mean protein concentration of 6.90 ± 0.95 g/dL (**Figure 4**). A 70% dilution with PBS alone significantly reduced total protein concentration to 4.51 g/dL ($P < 0.0001$). Hemodilution with PBS + 4 g% and 6 g% BSA reduced total protein concentrations to 5.75 g/dL ($P = 0.0169$) and 6.41 g/dL, respectively. A 90% dilution with PBS alone significantly reduced total protein concentration to 5.88 g/dL ($P = 0.0151$). Hemodilution with PBS + 4 g% and 6 g% BSA did not significantly alter total protein concentrations.

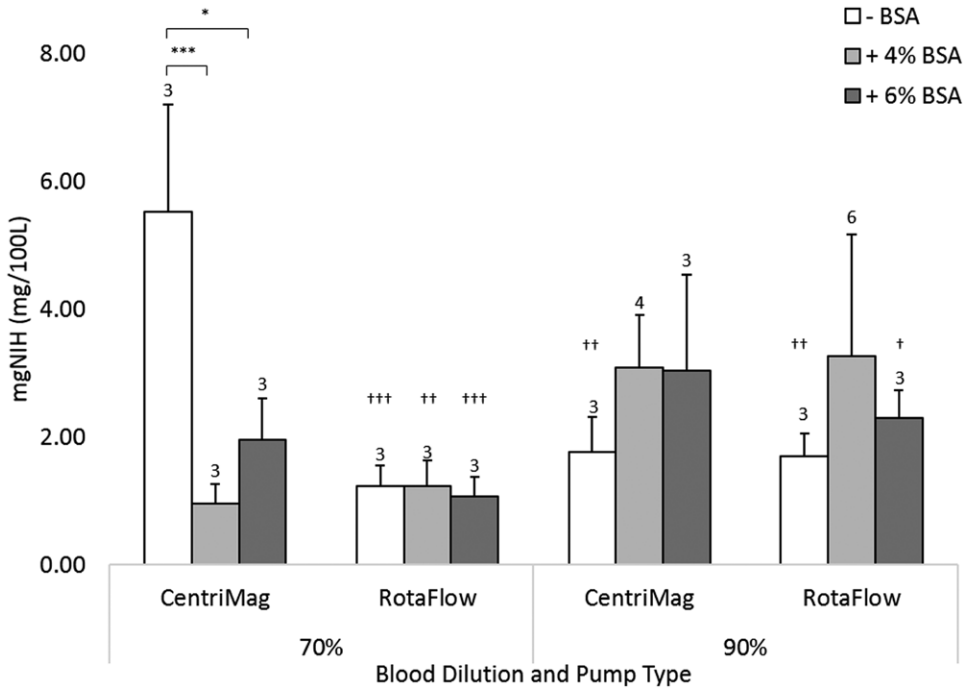

Figure 1. mgNIH values for combinations of 70% and 90% blood dilution. The CentriMag and RotaFlow pumps were tested, with and without BSA. The sample size is shown as data labels above the bars. Results are expressed as mean ± SD. †Significant compared with PBS 70% CentriMag. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. BSA, bovine serum albumin.

Impact of Diluents on Hemodilution as Defined in F1841

In the CentriMag device, the extent of hemolysis was dependent upon both the dilution and diluent. Higher volumes of PBS (within a 70–94% blood dilution) increased hemolysis and vice versa. Whole blood diluted to a 70–74% dilution with PBS resulted in significantly more hemolysis than 85–89% and 90–94% dilutions with PBS (**Figure 5A**; $P = 0.0073$; $P = 0.0032$, respectively). However, the reverse effect was observed

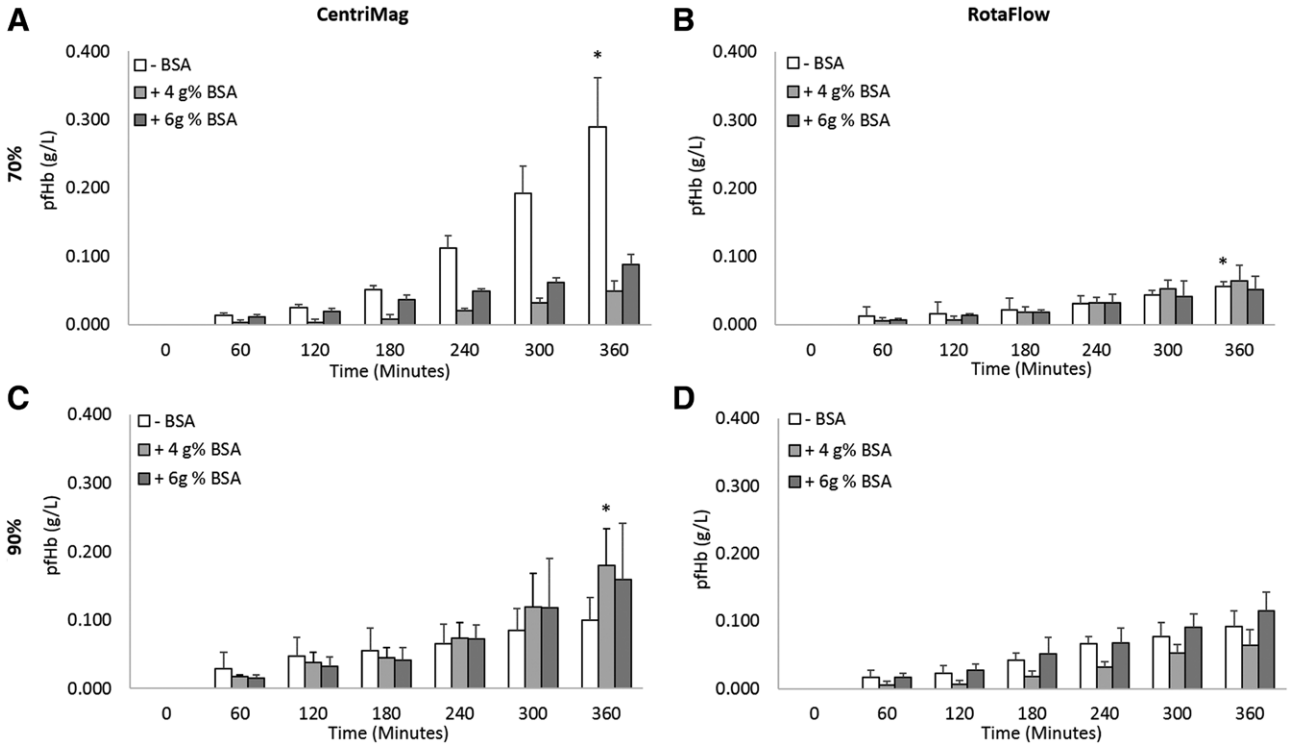

Figure 2. Plasma-free hemoglobin levels of 70% and 90% blood dilution. Release of pfHb overtime under 4 conditions using bovine blood: (**A**) CentriMag with a 70% dilution, (**B**) RotaFlow with a 70% dilution, (**C**) CentriMag with a 90% dilution, (**D**) RotaFlow with a 90% dilution. Results are expressed as mean ± SD. *$P < 0.05$.

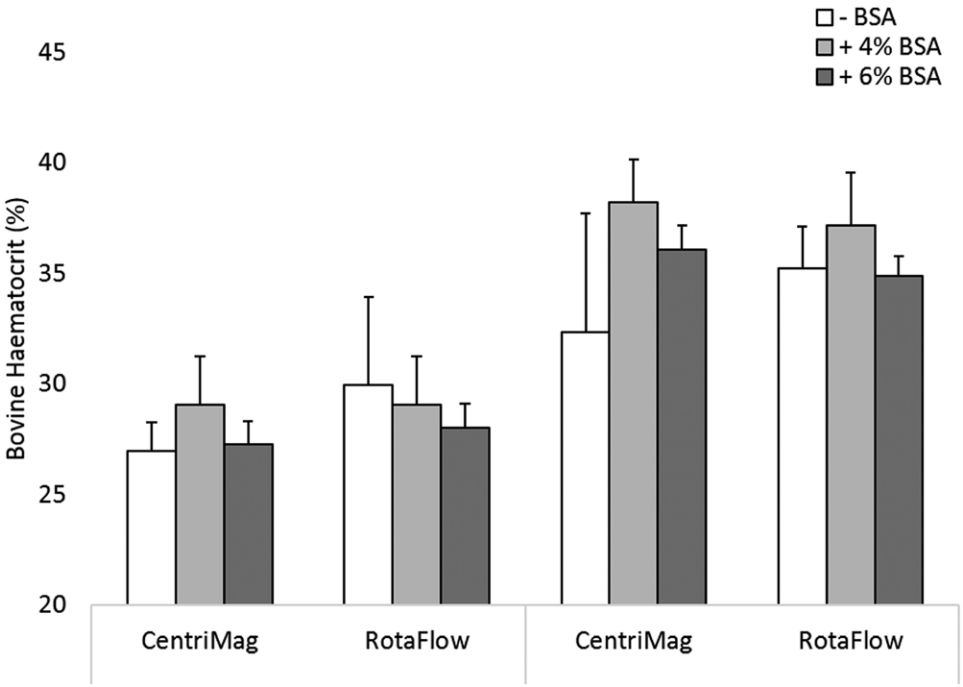

Figure 3. HCT values for the various combinations of 70% and 90% blood dilution. The CentriMag and RotaFlow pumps were tested, with and without BSA. Dotted lines represent a HCT 30% ± 2%. Results are expressed as mean ± SD. **** $P < 0.0001$. BSA, bovine serum albumin; HCT, hematocrit.

following hemodilution with PBS + 4 g% BSA, where higher volumes of PBS + 4 g% BSA decreased hemolysis, and vice versa, within a 70–90% range. Whole blood diluted to a 70–74% dilution with PBS + 4 g% BSA experienced less hemolysis than whole blood diluted to 85–90% with PBS + 4 g% (**Figure 5B**; $P = 0.0083$). A cutoff point, at which both mgNIH reaches the threshold, was observed at 83 ± 1% dilution (**Figure 5C**). Thus, bovine blood requiring <83% hemodilution should be diluted with PBS + 4 g% BSA and bovine blood requiring >83% hemodilution should be diluted with PBS alone (**Figure 6**).

ASTM Hemodilution with PBS + 4 g% BSA

Bovine blood was diluted with PBS + 4 g% BSA to reach an HCT of 30 ± 2%. Low hemolysis was achieved below a threshold of 2.0 mgNIH when tested in the CentriMag

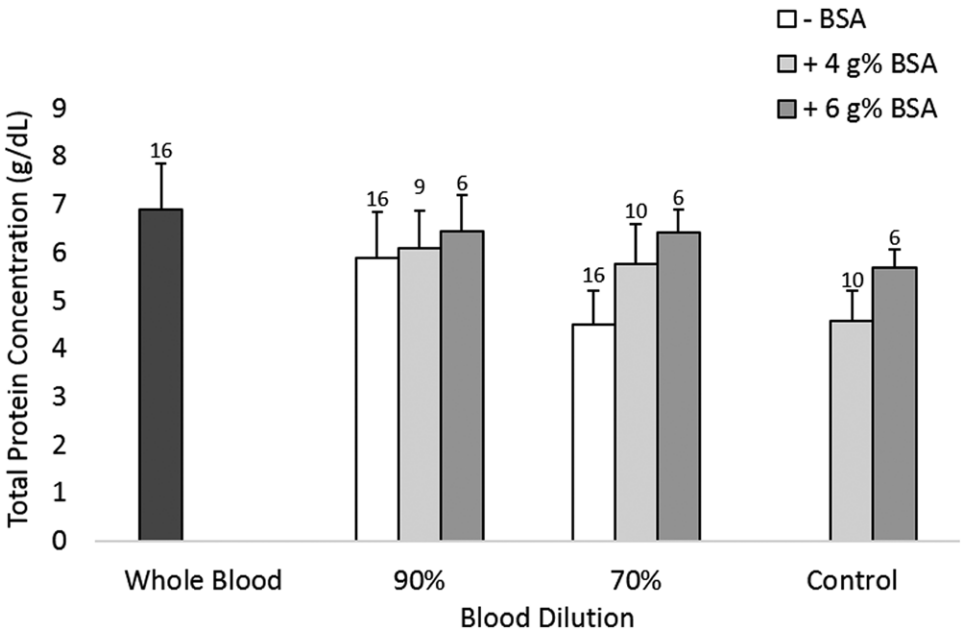

Figure 4. Total protein concentrations for the various combinations of 70% and 90% blood dilution. The CentriMag and RotaFlow pumps were tested, with and without BSA. Sample size shown as data labels above the bars. The dotted lines represent physiologic protein concentrations. Results are expressed as mean ± SD. †Significant compared with whole blood. ** $P < 0.01$, *** $P < 0.001$. BSA, bovine serum albumin.

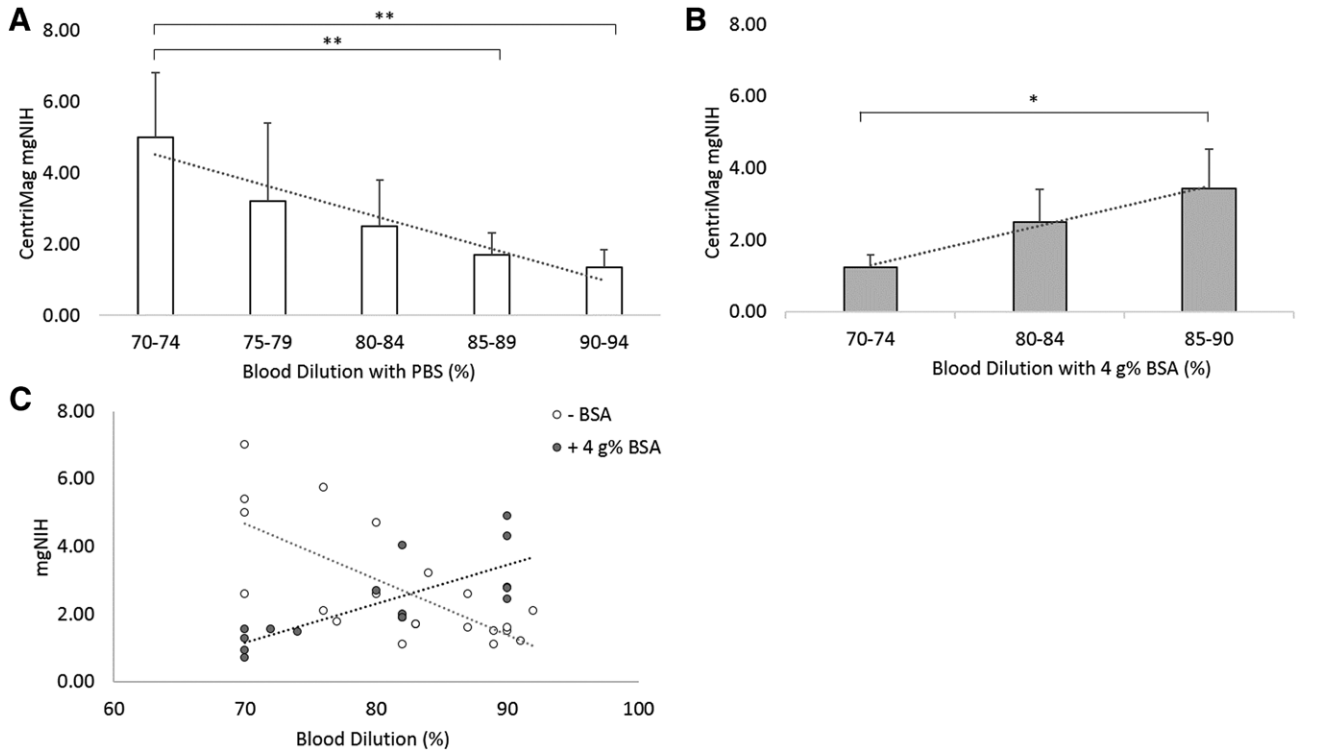

Figure 5. Hemolysis testing of dilutions and diluents. Hemodilution was carried out for a HCT value 30±2% with PBS or 4 g% BSA. (**A**) Tests without BSA (PBS alone), (**B**) tests with PBS+4 g% BSA, (**C**) individual tests from both diluents. Results are expressed as mean±SD. **P*<0.05, ***P*<0.01. BSA, bovine serum albumin; HCT, hematocrit; PBS, phosphate-buffered saline.

(1.4 mgNIH) and in the RotaFlow (1.5 mgNIH). There was no significant difference between devices (**Figure 7**).

Effects of Hemodilution Following VAD-Like Shear Stress in Human and Bovine Blood

Diluted bovine and human blood was subjected to VAD-like shear stress using the same donor under matched conditions (**Figure 8**). Hemolysis levels in bovine blood ($n=6$) were significantly reduced at a 70% blood dilution and significantly increased at a 90% blood dilution compared with PBS alone (**Figure 8**,A and B; $P=0.0313$). When bovine blood was diluted to achieve a HCT of 30±2%, hemolysis was significantly reduced compared with PBS alone (**Figure 8C**; $P=0.0313$). Human blood ($n=3$) shows similar alterations in hemolysis (**Figure 8**,D–F).

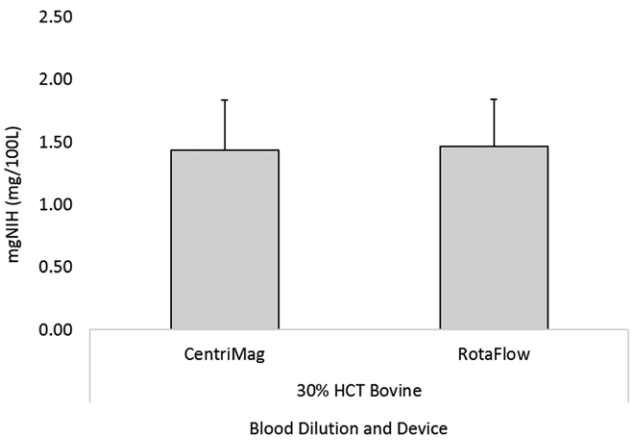

Figure 6. Process of hemodilution for *In Vitro* hemolysis testing. Whole blood should be diluted to achieve a standardized HCT while maintaining physiologic blood parameters, as per ASTM standards.

DISCUSSION

The purpose of this study was to investigate the effects of hemodilution during *in vitro* hemolysis testing with the CentriMag and RotaFlow devices. Previous studies have shown that dilution of washed RBCs with PBS can cause hemolysis.[9] However, this study is the first to describe the effect of hemodilution in a VAD test setting. As the F1841 standard recommends diluting donor blood with PBS, this study was carried out to identify whether diluting with PBS alone may be introducing bias when assessing hemolysis in bovine blood. Currently, there are no significant clinical differences between the devices; however, the safety profile of each device also depends on additional domains besides hemolysis that was not included in this study (*e.g.,* coagulation factors, platelets, leukocytes).

There may be multiple donor-donor variability factors that alter RBC mechanical fragility. However, this study introduced a method to remove dilution as a variability factor and prevent excessive hemolysis caused by hemodilution. As such, providing a way to view device-specific effects on hemolysis only, rather than a false level of hemolysis caused by hemodilution. To investigate the impact of dilution and diluents on hemolysis, *in vitro* testing was carried out using 90% and 70% blood dilutions. Levels of pfHb were measured to calculate the mgNIH, which accounts for the volume of blood, flow rate,

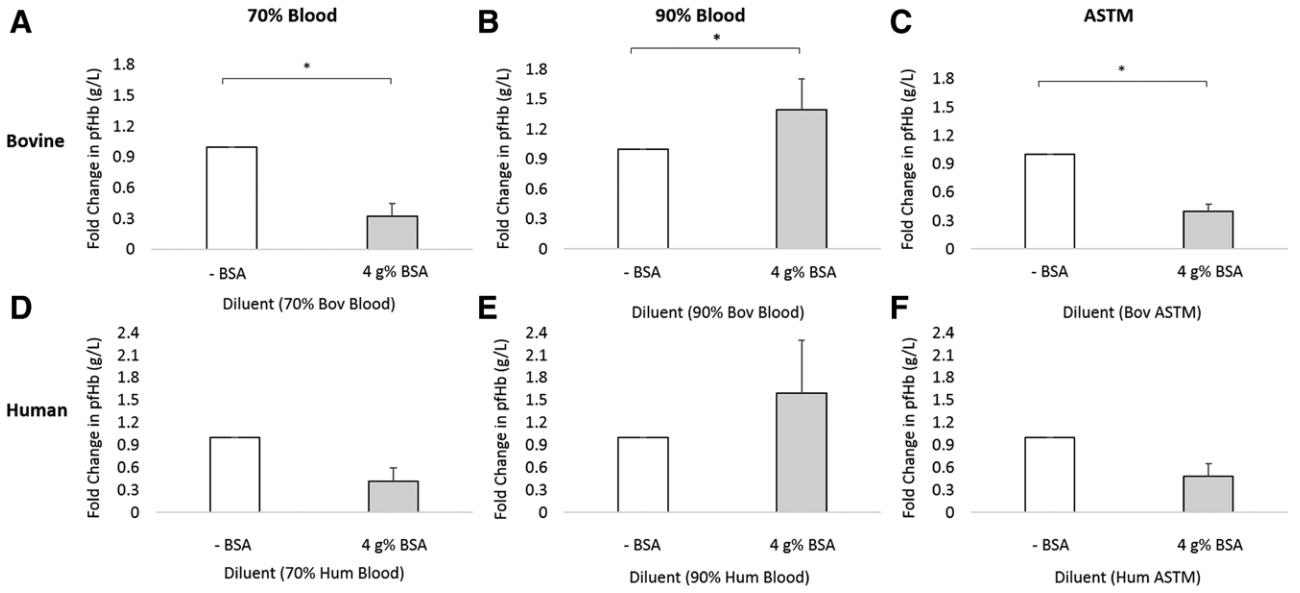

Figure 7. mgNIH verification for the CentriMag and RotaFlow pumps tested with PBS + 4 g% BSA. Hemodilution was carried out for a HCT value 30 ± 2% with 4 g% BSA. Results are expressed as mean ± SD ($n = 3$). BSA, bovine serum albumin; HCT, hematocrit; PBS, phosphate-buffered saline.

HCT, and time to normalize hemolysis. Background levels of pfHb were corrected for at the start of the test, and the subsequent increase in pfHb levels was measured. The mgNIH is more commonly used in publications and for benchmarking VADs. Therefore, this study used mgNIH for universal comparison of data and an acceptable limit for hemolysis was set at <2 mgNIH.

In the CentriMag, a 70% hemodilution with PBS caused high hemolysis, whereas blood with the same HCT diluted with PBS + 4/6 g% BSA showed significantly less hemolysis (**Figure 1**). As the only difference between the conditions was the addition of BSA, these results suggest that diluents alone affect hemolysis. Conversely, the mgNIH was unaffected by diluents using the RotaFlow (**Figure 1**). When PBS

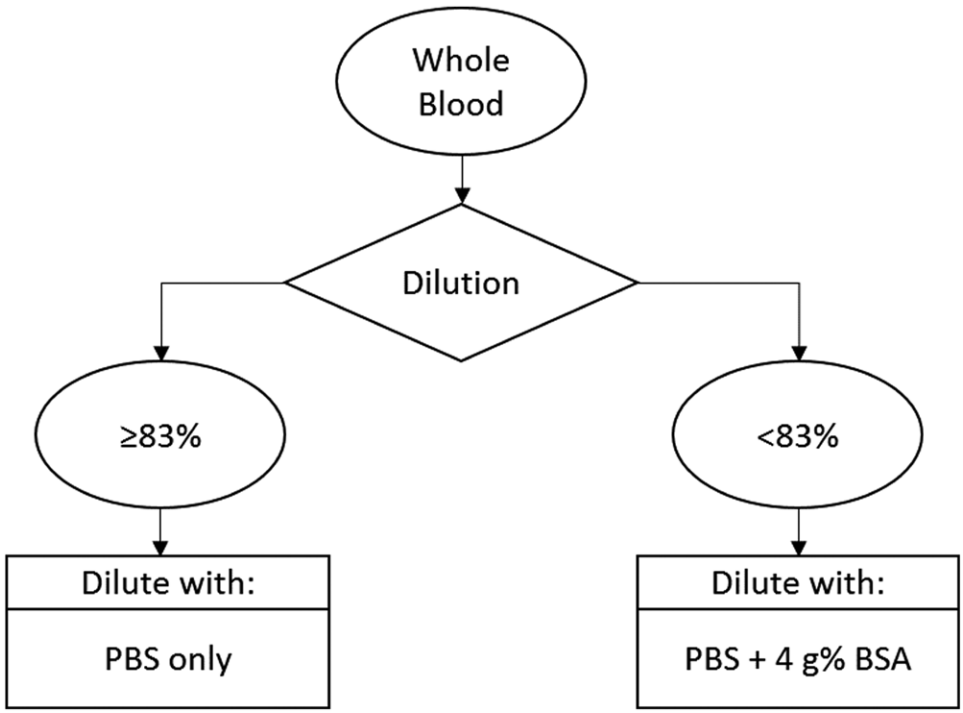

Figure 8. Hemodilution of human and bovine blood to 70% and 90% concentrations. Whole blood was diluted with either PBS or PBS + 4 g% BSA and subject to VAD-like shear (175 dyne/cm^2). Bovine blood ($n = 6$) was diluted to (**A**) 70% dilution, (**B**) 90% dilution, and (**C**) a HCT of 30 ± 2. Human blood ($n = 3$) was diluted to (**D**) 70% dilution, (**E**) 90% dilution and (**F**) a HCT of 30 ± 2. Results are expressed as mean ± SD. *$P < 0.05$. HCT, hematocrit; PBS, phosphate-buffered saline.

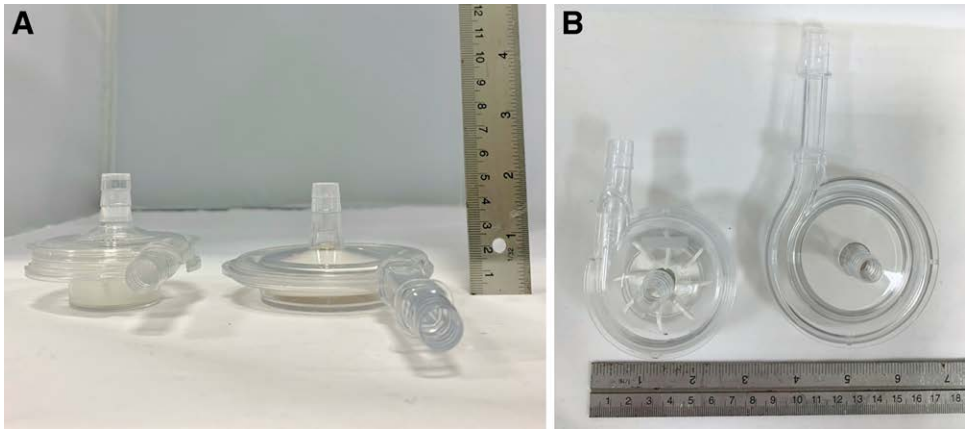

Figure 9. The CentriMag and RotaFlow pump heads. CentriMag (left) and RotaFlow (right) with a view onto the (**A**) pump outlet and (**B**) pump inlet.

is used alone, the RotaFlow produced lower hemolysis than the CentriMag, an observation shared by Wang *et al.*[25] As both devices underwent the same test conditions, these discrepancies may be attributed to a difference in device hemocompatibility. Both devices use a centrifugal pump design, but subtle differences exist. The CentriMag consists of an unshrouded, straight blade impeller design (**Figure 9**,A and B), which could cause blood recirculation,[26] whereas the RotaFlow consists of a shrouded spiral impeller design to optimize flow ratio with no stagnant zones.[27] Following these observations, the CentriMag may subject blood to increased residence time in higher shear flow regions. Furthermore, the impeller diameter of the RotaFlow is larger than that of the CentriMag (**Figure 9**,A and B). These differences result in the RotaFlow operating at a lower rotational speed to achieve the same fluid output as the CentriMag. The RotaFlow operated at 2,100 ± 41 rpm ($n = 21$) and the CentriMag operated at 2,363 ± 18 rpm ($n = 19$). Thus, the unshrouded design of the CentriMag and the higher operating speed could contribute to the observed higher hemolysis. As such, PBS + 4 g% BSA produced lower hemolysis levels in the CentriMag regardless of shear stress (**Figure 1**).

The data suggest a relationship between the volume of PBS and hemolysis (as PBS increases, hemolysis increases). Conversely, an inverse relationship was observed with PBS + 4 g% BSA, where increased volumes reduced hemolysis (**Figure 5**,A–C). However, a 90% blood dilution with PBS + 4 g% and 6 g% BSA caused higher levels of hemolysis in both devices compared with PBS alone. The increased hemolysis may be caused by the increased amount of albumin present. Thus, the protective effects of albumin may be concentration-dependent and damaging at higher levels. As such, mechanical fragility may also be influenced by protein concentration and oncotic pressure. Hemodilution with PBS + 6 g% BSA caused even higher hemolysis than PBS + 4 g% BSA, providing further evidence to suggest that albumin could be detrimental at higher concentrations (**Figure 1**).

This study also identified a cutoff point for dilution with PBS alone or PBS + 4 g% BSA (**Figure 5C**). Both diluents crossed the 2.0 mgNIH threshold at an 83 ± 1% blood dilution. Thus, regardless of initial HCT, to reach a standardized HCT and maintain a physiologic protein concentration in the blood, this study recommends the following: blood requiring <83% dilution should be diluted with PBS + 4 g% BSA, whereas blood requiring >83% dilution should be diluted with PBS only (as per **Figure 6**). The ASTM standard states that "acceptable ranges of physiologic blood parameters should be maintained before and during testing."[1] However, these standards only specify using PBS, but this diluent can produce results with increased hemolysis. Hemodilution with BSA would maintain protein levels within a physiologic range and reduce bias associated with high dilutions. As such, the use of PBS only would skew data due to unnecessary test fails. Previous research has shown that hemodilution with PBS alone increases RBC mechanical fragility and hemolysis.[9] Nevertheless, if minute differences exist between devices, these differences would be more evident at a 70% blood dilution with PBS than with PBS + 4 g% BSA. Consequently, dilution with PBS may introduce an unfair bias that results in exaggerated, unreliable data. The use of PBS + 4 g% BSA may remove this bias and reduce unnecessary hemolysis. However, a HCT of <42 would be predicted to fail if diluted in PBS + 4 g% BSA, due to higher levels of BSA, which is in contradiction to the requirements of the ASTM of maintaining physiologic blood parameters. As such, PBS alone is sufficient for hemodilution to maintain total protein concentrations at a 90% blood dilution (**Figure 4**). This research into the impact of diluents may result in alterations to the F1841 standard to include BSA for maintaining physiologic protein concentrations, where a larger amount of hemodilution is necessary. Thus, *in vitro* device testing may reduce variability and improve reproducibility between laboratories, regardless of dilution and HCT.

To identify any possible species and donor variability, additional testing was carried out with bovine and human blood using paired experimental conditions. The blood was subjected to VAD-like shear stress, and pfHb release was measured. The results matched those observed in the CentriMag testing (**Figures 1** and **8B**), thus, were not due to donor variability. The VAD-like shear device was used in this study due to the advantage of experimentation with small quantities of human blood. Furthermore, experimentation was able to be carried out using multiple diluents, concentrations, and donors at any 1 time. In contrast, the CentriMag device may only test a single donor, diluent, and concentration. However, the main limitation of the VAD-like shear device is the continuous, long-exposure time. Whereas blood is commonly exposed to shear stress within a VAD from 1–50 ms at a time.[28] Nevertheless,

hemodilution with PBS and BSA exerted the same effects on human blood (**Figures 1** and 8D–F), suggesting that patients undergoing hemodilution may experience similar effects when exposed to shear stress. Such conditions occur during cardiopulmonary bypass surgery, which employ the use of extracorporeal circulation and hemodilution with 0.9% sodium chloride solution (normal saline).[29,30] Therefore, the use of saline may contribute to higher levels of hemolysis by increasing RBC mechanical fragility and worsening potential clinical outcomes such as thrombus formation and organ failure.[31]

CONCLUSION

This study showed that hemolysis occurred more often following large dilutions with PBS than PBS + 4 g% BSA. Thus, hemodilution using PBS alone may be a cause of hemolysis. Blood diluted to 70% with PBS in the CentriMag resulted in high mgNIH. Research suggests that RBCs possess a surface coating, which provides a natural protective layer against shear stress. Hemodilution with PBS may modify this coating by reducing physiologic protein levels, such as albumin, and increase mechanical fragility. Subsequently, the addition of PBS + 4 g% BSA to diluted blood was investigated as a method of maintaining the physiologic protein concentration. For a blood dilution of 70%, hemodilution with PBS + 4 g% BSA resulted in a <2 mgNIH. BSA may prove useful to normalize the harmful effect of PBS dilution during *in vitro* hemolysis testing. However, a 90% blood dilution with PBS + 4 g% BSA caused higher hemolysis than dilution with PBS. Thus, BSA may prove harmful when used at high blood dilutions. This study recommends how to dilute blood according to the dilution required to observe device-specific effects and avoid unnecessary hemolysis. Donor blood requiring larger dilutions (<83%) require dilution with PBS + 4 g% BSA, whereas blood requiring smaller dilutions (>83%) require dilution with PBS alone. When needed, the inclusion of BSA maintains physiologic protein concentrations and protect RBCs from enhanced mechanical fragility. Therefore, the inclusion of BSA may remove the bias caused by large blood dilutions with PBS and gain a true mgNIH. As such, this study aids the development of *in vitro* hemolysis testing by maintaining physiologic blood parameters and reducing both bias and variability between tests.

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