Original paper

Perfusion

Perfusion
2016, Vol. 31(5) 401–408

DOI: 10.1177/0267659115615206
prf.sagepub.com
SAGE

Hemolysate-mediated platelet aggregation: an additional risk mechanism contributing to thrombosis of continuous flow ventricular assist devices

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Abstract

Despite the clinical success and growth in the utilization of continuous flow ventricular assist devices (cfVADs) for the treatment of advanced heart failure, hemolysis and thrombosis remain major limitations. Inadequate and/or ineffective anticoagulation regimens, combined with high pump speed and non-physiological flow patterns, can result in hemolysis which often is accompanied by pump thrombosis. An unexpected increase in cfVADs thrombosis was reported by multiple major VAD implanting centers in 2014, highlighting the association of hemolysis and a rise in lactate dehydrogenase (LDH) presaging thrombotic events. It is well established that thrombotic complications arise from the abnormal shear stresses generated by cfVADs. What remains unknown is the link between cfVAD-associated hemolysis and pump thrombosis. Can hemolysis of red blood cells (RBCs) contribute to platelet aggregation, thereby, facilitating prothrombotic complications in cfVADs? Herein, we examine the effect of RBC-hemolysate and selected major constituents, i.e., lactate dehydrogenase (LDH) and plasma free hemoglobin (pHb) on platelet aggregation, utilizing electrical resistance aggregometry. Our hypothesis is that elements of RBCs, released as a result of shear-mediated hemolysis, will contribute to platelet aggregation. We show that RBC hemolysate and pHb, but not LDH, are direct contributors to platelet aggregation, posing an additional risk mechanism for cfVAD thrombosis.

Keywords

hemolysis; thrombosis; ventricular assist devices; platelet aggregation; lactate dehydrogenase (LDH); multiplate analyzer

Introduction

Continuous flow ventricular assist devices (cfVADs) have emerged as standard-of-care therapeutics, restoring hemodynamics for advanced heart failure patients, either as a bridge-to-transplant or destination (long-term) therapy.[1,2] Despite their clinical success, cfVADs remain plagued by thrombosis and hemolysis, resulting in pump malfunction, recurrent heart failure, neurologic events, embolic complications and possible death. In an attempt to limit thrombosis, cfVAD patients require life-long antiplatelet and anticoagulation regimens. Inadequate or ineffective anti-thrombotic drug regimens combined with high pump speed, perturbed flow and high shear stress often result in red cell hemolysis and subsequent thrombosis. As such careful management of anti-thrombotic regimens and trending of specific thrombosis- or hemolysis-associated biomarkers may help minimize adverse prothrombotic events and post-implant complications.

Over the past year an increase in cfVAD thrombosis has been observed at multiple centers.[3] These investigators report that the development of pump-related hemolysis and a rise in LDH were noted to presage throm-

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Presented at the 36th Annual Seminar of the American Academy of Cardiovascular Perfusion, San Antonio, TX, 5–8 February 2015.

botic events. Similarly, the Interagency Registry for Mechanically Assisted Circulatory Support (INTERMACS) reported an increase of pump exchange or death due to pump thrombosis during 2011 and 2012, where elevated LDH in the first month post-implant was noted to be a strong predictor of pump thrombosis.[4] In previous work, we and others have demonstrated and further defined the role of non-physiological flow and elevated shear stresses as to their contribution to platelet aggregation and activation.[5–7] Similarly, these perturbed flows may lead to hemolysis and the release of RBC contents, i.e., LDH and plasma free hemoglobin (pHb). What remains unknown is the link between cfVAD-mediated hemolysis and pump thrombosis. Are RBC released contents a risk factor or a risk maker for predicting adverse prothrombotic events in patients implanted with cfVADs? In the present study, we hypothesized that elements of RBCs, released as a result of shear-mediated hemolysis, will contribute to platelet activation. As such, we specifically examined the effect of adding RBC hemolysate (RBC-hemo), pHb and LDH to platelets as to their subsequent impact on platelet aggregation, using impedance aggregometry.

Methods

Sample preparation and material collection

Fresh human blood was collected from healthy adult volunteers (n = 4) via venipuncture and mixed with 10% (v/v) anticoagulant citrated dextrose – Formula A (ACD-A). Blood was collected only from those donors free of alcohol consumption the night before or caffeine the morning of blood donation to maximize platelet counts and prevent desensitization of the platelets. All volunteers provided informed consent for the study which had University of Arizona Institutional Review Board (IRB) approval.[8] Blood samples were centrifuged to separate platelet rich plasma (PRP) from white and red blood cells (RBCs). The RBCs were washed (x3) in PLT buffer (Hepes-modified $Ca2^{+}$-free Tyrode's buffer) via centrifugation and counted using a Z2 Particle Counter (Beckman Coulter, Miami, FL). Washed RBCs (5 $x10^{6}$ cells/μl) were subjected to high frequency mechanical sonification (Branson Sonifier SLP, Atkinson, NH) at 60 W, 20 kHz for 5 minutes to generate RBC-hemo. Platelets were also counted using a Z2 Particle Counter (Beckman Coulter) and normalized to 1.5 x 10^{5} platelets/μl with PLT buffer for aggregation studies using a Multiplate Analyzer (Roche Diagnostics, Mannheim, Germany). To examine the effect of hemoglobin (Hb) and LDH on platelet aggregation, these compounds were added exogenously to PRP, as outlined below. Lactate dehydrogenase 1 (LDH, Lee Biosolutions, St. Louis, MI) and human plasma free hemoglobin (pHb, MyBioSources, San Diego, CA) were purchased for the study. Plasma free hemoglobin (pHb) was solubilized in normal saline to achieve a stock of 6 mg/ml and diluted for use, with concentrations verified using a NanoDrop 2000 C UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). pHb was studied at a concentration range of 2 – 120 mg/dl. Stock LDH was obtained as a solution with an enzymatic activity of 740 IU/ml. LDH was studied at a concentration range of 200 – 1800 U/L.

Platelet aggregation study

RBC-hemo, LDH and pHb as test reagents were added to platelets (PRP) at defined concentrations. The PRP + test reagent mixtures were incubated at 37^{O}C for 3 min. Platelet aggregation was then measured utilizing the Multiple Analyzer (MA) (Figure 1A) - an impedance aggregometer that measures the increase in electrical impedance across two metal wires in a test cell when platelets accumulate and aggregate in response to an agonist (Figure 1C, D). The MA has 5 channels, each one containing a single-use test cell with two independent sensor units (Figure 1C).[9] The change of impedance, or resistance, is proportional to the amount of platelets sticking to the electrodes and is continuously detected from each sensor unit separately, transformed to arbitrary aggregation units (AU) and displayed as two aggregation curves. Aggregation is plotted against time. The maximum height of the curve represents the aggregation level while the curve slope (Figure 1B) represents the reaction velocity. The most important parameter is the area under the aggregation curve (AUC) or AU*min.[10]

Aggregation experiments were performed exposing PRP to different agonists. Each experiment was repeated four times using PRP (150,000 platelets/μl) obtained from different donors. In the first experiment, PRP was exposed to different dilutions of RBC hemolysate: 100%, 40% and 20%. After 12 minutes, 5 μM of adenosine diphosphate (ADP) was added to each channel and the test continued for another 18 minutes to validate and verify the viability and optimal aggregation of the tested platelets. Two control samples were used: PRP without any agonist and intact RBCs (washed RBCs). Similarly, we tested pHb at 2, 20, 40 (cfVAD-induced threshold), 80 and 120 mg/dl and LDH at 200, 600 (cfVAD-induced hemolysis threshold), 1200 and 1800 IU/L.

Scanning electron microscopy analysis

Aggregated or control (unactivated) platelets (150 μL) were added to 150 μL of 2% v/v glutaraldehyde in platelet buffer, pH 7.4 and simultaneously placed onto 12-mm circular glass coverslips in 24-well plates for 15 minute. Excess solution was partially aspirated, leaving approximately 50 μL of mixture behind. The coverslips were

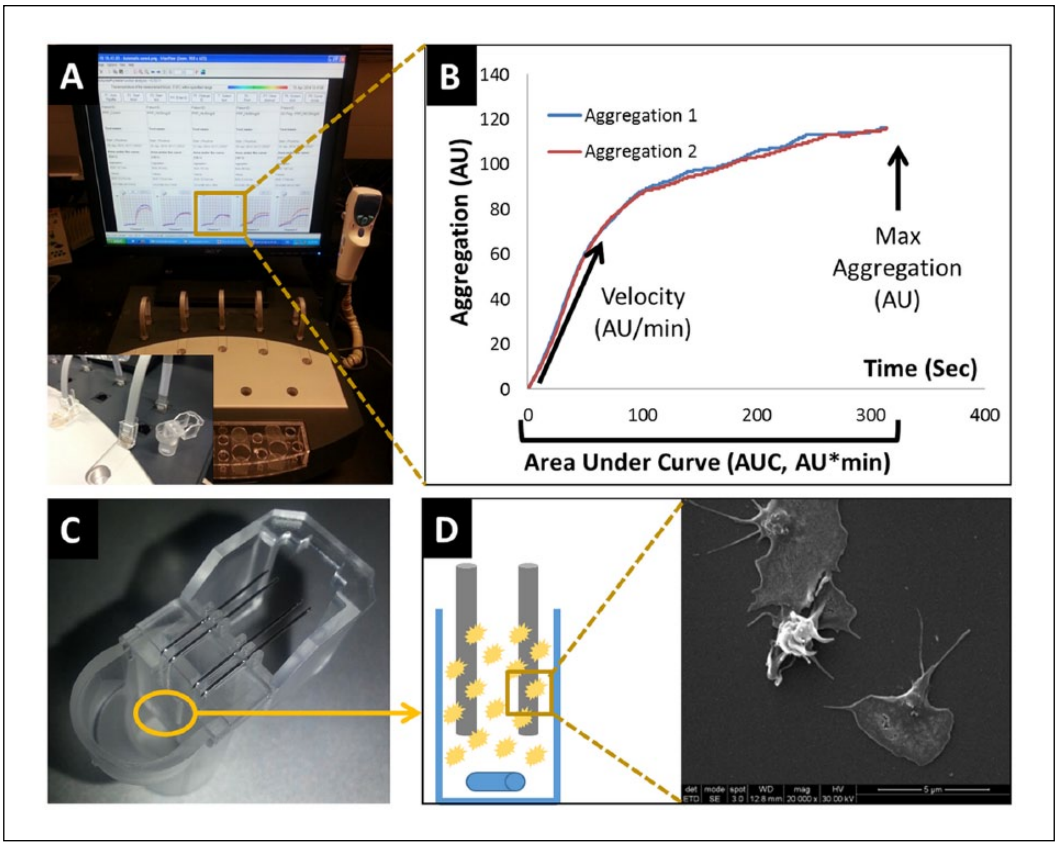

Figure 1. Multiplate Analyzer Impedance Aggregometry. A) Multiplate Analyzer console. B) Example of an aggregation measurement with 6 minute runtime. C) Multiplate test cells. D) Platelets aggregate to electrodes, thus, increasing the electrical impedance.

washed with 25%, 50%, 75% and 100% of double-distilled H_2O in 1% glutaraldehyde. The coverslips were then dehydrated through a graded ethanol series of 0%, 25%, 50%, 75% and 100% in double-distilled H_2O. Samples were stored in 100% ethanol until critical point, drying through a series of 25%, 50%, 75% and 100% hexamethyldisiloxane (HMDS) in ethanol exposures. Each preparation stage required a 5-min immersion at room temperature. The coverslips were then mounted on double-sided carbon tape, allowed to air dry overnight and then sputter-coated with islanded gold in an argon chamber. Samples were imaged at 15 kV with an aperture spot size of 3 by using an Inspect F50 scanning electron microscope (FEI, Hillsboro, OR) to identify platelet morphological changes, which represent platelet activation and platelet aggregation.

Statistical analysis

Statistical tests were performed using Microsoft Excel v2010. Single comparison t-tests were used in two-population comparisons. Differences were considered significant if $p<0.05$.

Results

Effect of the addition of RBC components on platelet aggregation

The addition of RBC hemolysate to PRP resulted in a definitive increase in platelet aggregation versus control (Figure 2). Further, when different dilutions of RBC hemolysate (100%, 40% and 20%) were added to PRP, a concentration-dependent behavior as to this augmentation of platelet aggregation was observed (Figure 2A-blue). No aggregation was measured for the controls of PRP alone or the addition of washed intact RBCs. After 12 minutes of measurement, ADP was added to drive aggregation to a functional maximum (Figure 2A-red, 2B) to validate and verify platelet viability and functionality.

The addition of LDH to PRP, however, did not increase platelet aggregation, even with concentrations three times higher than the typical clinical LDH hemolysis threshold level of 600 IU/L (Figure 3A). Plasma free hemoglobin showed little to no aggregation at a physiological concentration (2 mg/dL), but did increase aggregation at higher concentrations (normal serum

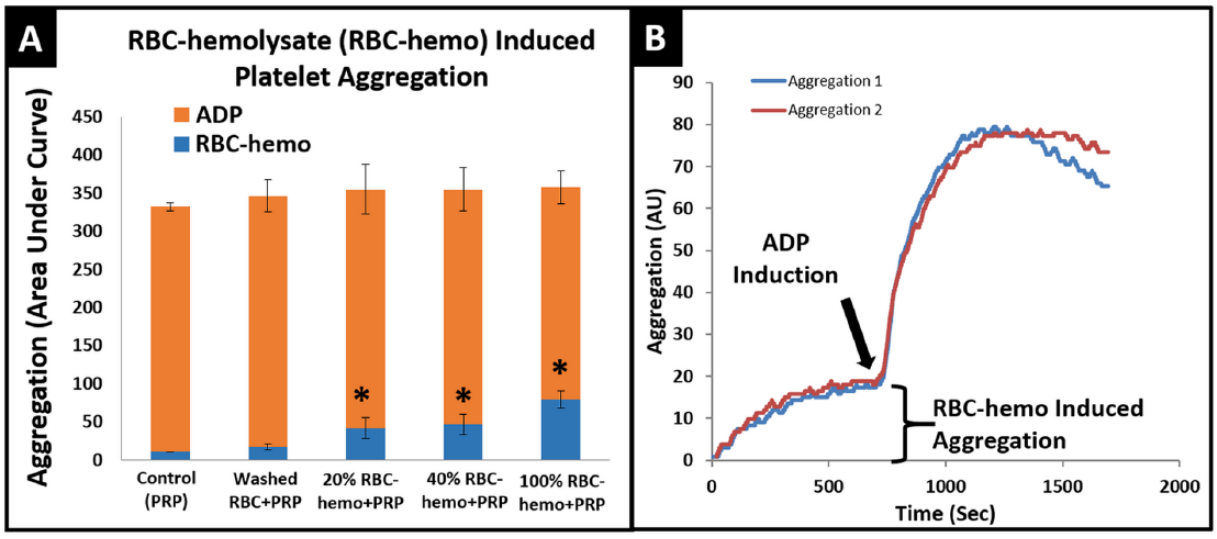

Figure 2. Platelet aggregation by red blood cell hemolysate (RBC-hemo) and adenosine diphosphate (ADP). A) Partial platelet aggregation induced by RBC-hemo and maximal platelet aggregation mediated by the addition of ADP. All aggregation induced by RBC-hemo are significant from control, which has only clean PRP. *p-value <0.05. B) Example of a single experiment showing spontaneous aggregation measured within 12 minutes then, subsequently, induced by ADP to demonstrate platelet viability and functionality. n-samples = 4.

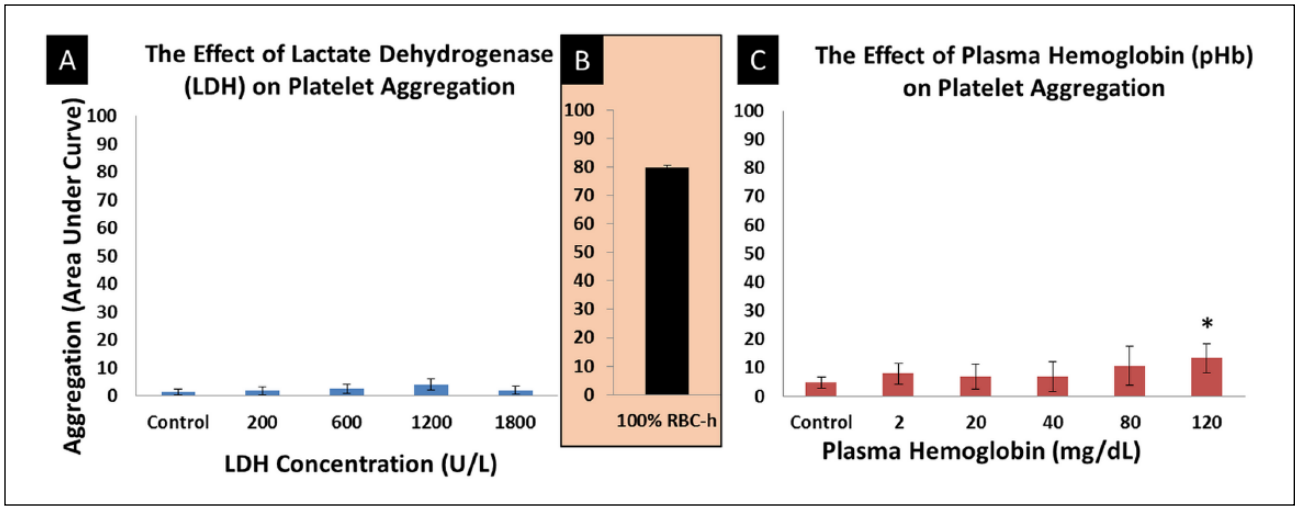

Figure 3. Platelet aggregation by lactate dehydrogenase (LDH) and plasma free hemoglobin (pHb). A) Platelet aggregation induced by various concentration of LDH showing no effect. B) Platelet aggregation mediated by red blood cell hemolysate (RBC-hemo) extrapolated from Figure 2 for comparison purposes. C) Platelet aggregation induced at various pHb concentrations showing a significant increase with 120 mg/dL when compared to the control sample, which has no pHb. *p-value <0.05. n-samples = 4.

threshold value of 40 mg/dL), with progressive increases to 120 mg/dL (Figure 3C).

Effect of the addition of RBC components on platelet activation and morphology

RBC-hemo generated from washed RBCs (5 x10^6 cells/µl, Figure 4B) significantly aggregated platelets to an extent far greater than that observed with exposure to both LDH and pfHb at concentrations typically associated with cfVADs *in vivo* in implanted patients (i.e., LDH (600 IU/L) and pHb (40mg/dL),(Figure 4A) as reported in the Interagency Registry for Mechanically Assisted Circulation Support (INTERMACS)).[4,11,12] Scanning electron microscopy (SEM) analysis of the platelets aggregated by RBC-hemo showed significant morphological

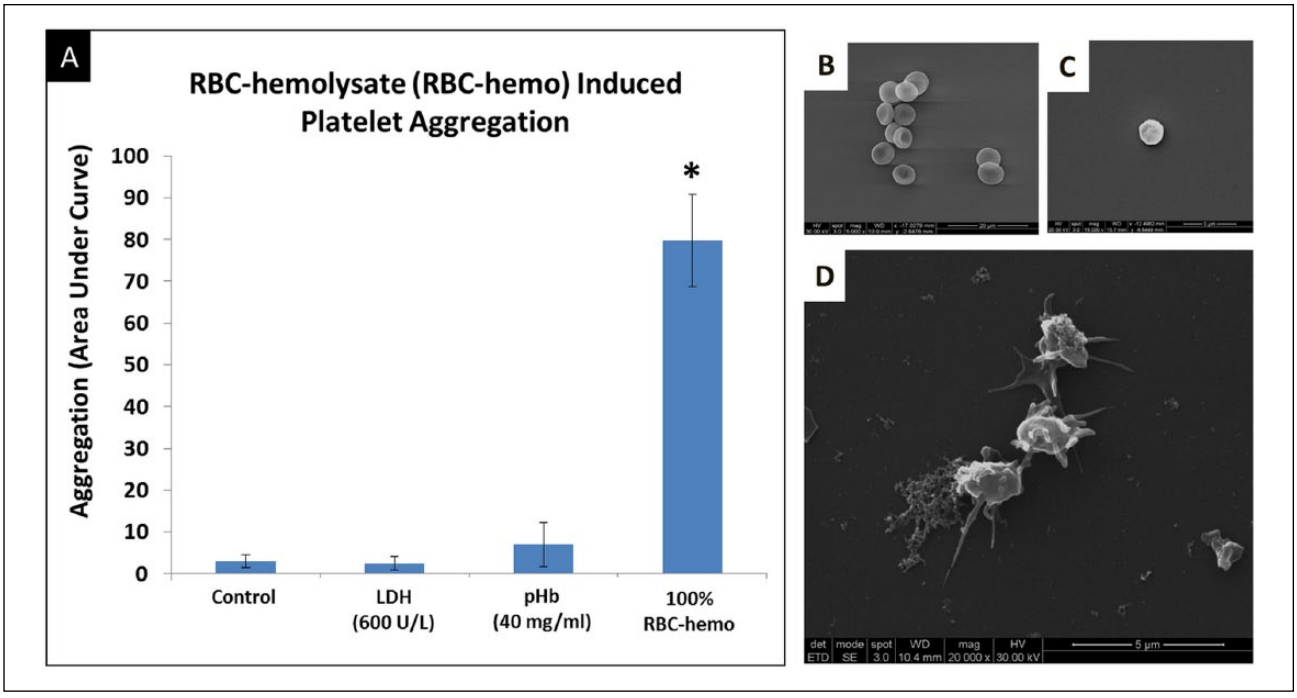

Figure 4. Prothrombotic activity of RBC hemolysate. A) Platelet aggregation effectiveness of RBC hemolysate in comparison to that of LDH and pHb (at levels observed in cfVAD patients); RBC-hemo demonstrated at least a 7-fold increase in aggregation when compared to pHb and 15-fold when compared to LDH and control (PRP alone). *p-value <0.05. B) Scanning electron microscope (SEM) of RBC. C) SEM of non-activated platelets. D) SEM of RBC-hemo-induced platelet aggregation. n-samples = 4. * p-value <0.05.

change, with pseudopods connecting to each other (Figure 4D), when compared to control platelets from normal volunteers (Figure 4C).

Discussion

In this study, we examined the effect of exogenous addition of mechanically damaged RBCs and specific RBC constituents on platelet aggregation and activation. Our results clearly show that RBC hemolysate and pHB, added to platelets at the concentrations tested, led to enhanced platelet aggregation and activation. In contrast, LDH, a clinical marker of hemolysis, did not contribute mechanistically to platelet activation or aggregation.

The studies presented here highlight a potential mechanistic link between cfVAD-induced RBC hemolysis and thrombosis. We found that RBC hemolysate effectively and significantly activated platelets (Figure 2) in a concentration-dependent manner (Figure 2A-blue). This raises the possibility that the potential for pump thrombosis clinically might be closely associated with the level of hemolysis *in vivo*.

RBC hemolysate contains numerous constitutive elements that may induce platelet aggregation and activation. To consider the mechanism, it is possible that RBC contents, membrane fragments or a combination of both act as agents stimulating platelet activation or provide a substrate for enhanced aggregation. RBC composition has been studied extensively over the years, with summary findings of protein being 49%, lipids 46% and carbohydrates, other metabolites and ions as the remainder of the constituents.[13,14] Beyond the dominant RBC protein – i.e., Hb, which we herein and others[12,15,16] have demonstrated to lead to enhanced platelet activation and aggregation, other RBC proteins as well may drive aggregation. Recent studies of the RBC proteome have now found >750 protein constituents in RBCs.[17,18] Proteomic analysis has demonstrated many functional classes of proteins in RBCs, including kinases, glycoproteins, adhesion proteins, transporters and proteases, all of which are recognized as having pro-thrombotic members within each class.[17] RBCs also contain many strong prothrombotic metabolic constituents, including ADP[19–21] and calcium.[22] Further, RBC membranes contain a range of lipids, including a predominance of phospholipids and cholesterol, with small amounts of glycolipids. Lipids are asymmetrically distributed in RBC membranes, with most choline-containing phospholipids found in the outer monolayer whereas most aminophospholipids are found in the inner membrane.[23,24] Aminophospholipids, such as phosphatidylserine and phosphatidylethanolamine, being negatively charged, have been demonstrated to be prothrombotic.[25] With

hemolysis, these inner membrane elements are effectively everted, allowing direct exposure to platelets, posing a prothrombotic risk. Further, phosphatidylserine within membrane fragments has been postulated to regulate thrombin release.[26] Therefore, to further narrow down mechanistic contributors, individual hemolytic components should be further characterized and investigated as to their contribution to cfVAD-induced hemolysis and thrombosis.

Our observations of total RBC hemolysate-enhanced platelet aggregation and our findings of the pro-activation and aggregation effects of hemoglobin are consistent with and supported by the reports of both Iuliano et al.[27] and Villagra et al.[16] Iuliano examined the effect of adding either supernatants derived from "stirred erythrocytes" or exogenous hemoglobin on platelet aggregation, utilizing PRP and light aggregometry. They observed that stirred supernatant, i.e., RBC contents derived from mild to moderate mechanical damage, containing hemoglobin, as well as direct exogenous hemoglobin exposure, both led to enhanced platelet aggregation. Similarly, Villagra et al. examined the effect of cell free hemoglobin on the activation of platelets in the form of PRP, with activation determined based upon gpIIb/IIIa and p-selectin expression. They similarly demonstrated a dose-dependent increase in platelet activation with Hb exposure. Our studies go beyond these studies. Here, we show that Hb can activate platelets which are in a more pure form, i.e., as purified cells, namely, as gel-filtered platelets, free of association with plasma proteins, which may have additive effects on both activation and aggregation.

The recent work of Helms et al. further supports our findings of the connection and role of hemolysis as a driver of platelet activation.[15] Their studies attempted to dissect which intra-RBC constituents induce or amplify platelet activation, observing, similar to our study, that whole RBC hemolysate, despite slight preparation differences, drives platelet activation when added to PRP. They further showed that hemoglobin alone activates platelets, consistent with our findings. Interestingly, they observed that, when apyrase, a nucleotide di- and tri-phosphatase, was added to dialyzed hemoglobin, a diminution of the extent of the activation of platelets was observed, compared to untreated control, arguing for a dominant role of hemoglobin-bound ADP as a major actor in hemolysis-mediated activation. Their observations and our finding remain congruent in that: 1. even with exposure of PRP to apyrase-treated dialyzed Hb, platelet activation was still observed to increase over baseline, 2. our Hb treatment involved exposure of PRP to highly purified reagent grade Hb free of small molecules such as ADP with observed significant activation, 3. Iuliano, as well, demonstrated that exposure of PRP to Hb led to enhanced platelet activation, with or without apyrase admixture, implying a direct role for Hb independent of concomitant ADP.[27]

Scanning electron microscopy analysis of platelets exposed to RBC hemolysate showed high levels of aggregation and activation as evidenced by extruding filopods, adhesion and irregular morphology when compared to inactivated platelets (Figure 4). The finding is congruent with the findings of Kuwahara and colleagues where platelets change shape[28] and platelets interact with other platelets forming aggregates.[29]

On the clinical level, in addition to pHb, the level of hemolysis can be identified by the use of LDH as a clinical biomarker.[16,30,31] In fact, INTERMACS has established two criteria for defining hemolysis as adverse events: 1) LDH above 600 IU/L or 2.5 times the upper limit of laboratory normal and 2) plasma free hemoglobin above 40 mg/dL with signs/symptoms.[4,12] As was a starting goal of this study, we have clarified the role of LDH, establishing that it does not aggregate platelets (Figure 3), suggesting that it is solely a risk marker. In contrast, as discussed above, while we found that pHb activated platelets, our findings show that it does so at a concentration three times higher than the hemolysis criteria defined by INTERMACS. This finding is also consistent with Helms et al.[15] and previous studies,[16,27] as previously stated. As such, the level of LDH and pHb are, therefore, best utilized for trending cfVADs-induced hemolysis rather than for firm pump thrombosis diagnosis. Such needs require clinical tests, including an analysis of pump power consumption, ramping studies, echocardiography and radiological imaging studies.

The time sequence and initiating events in cfVAD thrombosis remain a challenge and a paradox, to a degree. It is a bit of a "chicken and the egg situation" in that it remains unclear whether pump thrombosis leads to RBC hemolysis, further potentiating platelet activation and thrombus formation or if vice versa cfVADs hemolyze RBCs, thus, amplifying platelet activation. It still remains to be investigated. What we now know, in conjunction with previous studies,[15,16,20,21,32,33] is that RBC hemolysate mediated platelet aggregation, but not LDH and marginal platelet activation occurs with high plasma free hemoglobin levels. Further understanding of the mechanism of platelet activation via RBC hemolytic elements may provide additional pathways to limit VAD (shear)-mediated platelet activation and reduce the thrombotic risk for VAD patients.

Acknowledgements

We thank the DiaPharma Group Inc., in particular, Dr. Stephan Delaney, for the provision of the Multiplate Analyzer.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This project was partially funded by a Quantum Grant from the NIH/NIBIB (grant #5U01EB012487) and CARIPLO Foundation (grant #2011-2241 CARIPLO).

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Discussion: Hemolysate-mediated platelet aggregation: an additional risk mechanism contributing to thrombosis of continuous flow ventricular assist devices

Presenter **Phat L Tran**

MR. ERIC JENKINS (Dexter, Michigan): In the practical application of your science described, did you find, in your research, a correlation between the level of hemolysis and thrombosed devices?
MR. TRAN (Tucson, Arizona): Yes. There is always a level of hemolysis. At the University of Arizona, we measured lactate dehydrogenase (LDH) levels. We also have a suspicion when we see some redness in the patient's urine; then, we will measure the plasma free hemoglobin. There is definitely a correlation and there is a large amount of data on those clinical findings. There are other mechanical devices that also cause hemolysis. I hope I have convinced the audience that there is more than just the hemolysis to consider; there is also the shear factors involved. For every well-intended purpose, it seems that there is always a good and a bad outcome. So, it is up to us to deliver and attempt to counteract that adverse outcome.
MR. JENKINS: Is there a threshold at which you start making clinical changes in pump or patient management?
MR. TRAN: Yes. INTERMACS has published a report showing a threshold level of 600 international units of LDH, which would be the threshold level. If you go beyond that, then there is an indication of thrombosis. There are also thresholds for plasma free hemoglobin and LDH, which we referenced in the paper.
MS. LINDA MONGERO (Locust Valley, New York): If you take this now into the clinic, do you think there is an opportunity to filter these ghosts or administer some kind of platelet inhibitor?
MR. TRAN: Yes. I will first address the question with what is going on, to my best knowledge, from the clinical side. We do use other anti-platelet drugs sometimes. We have used eptifibatide or dipyridamole. The ventricular assist devices (VADs) are placed completely internally, so we cannot filter the red blood cells in this case. Hopefully, the patient has good functional organs that can do that for us, so that is why, many times before implantation of a VAD, the health care team needs to confirm that the patient has all of his/her organs intact. If not, that would be a red flag for the patient who has poor renal function, renal failure or liver function that is not intact. In the laboratory, we have looked at ways to better prevent platelet activation and aggregation. There is a DMSO chemical agent, dimethyl sulfoxide, that we use, which can modulate the membranes of platelets. Now, if you think about it, this is more like a tall building with rubber in its foundation at the bottom that can withstand an earthquake and absorb shock. By modulating the membrane on the platelet to make it more fluidic, you can absorb more shock and more shear stress induced by the device. We have much good data, hopefully, that we can convince peer reviewers to publish, that we can inhibit platelets at high levels of shear stress. We are talking about high perceived levels up to 20 to 30 percent.
CO-MODERATOR RICHARD MELCHIOR (Philadelphia, Pennsylvania): I want to include one question from the webcast audience. Mr. Matt Tindal from Birmingham, Alabama, has a question as follows: Do you think this would be applicable for monitoring thrombosis during extracorporeal membrane oxygenation (ECMO)?
MR. TRANS: Yes, absolutely. In ECMO circuits, you have an oxygenator to trap clots. We have observed, clinically, when using the CentriMag®, where you do not have an oxygenator, problems with thrombi in the connectors that dislodge and cause strokes in patients.