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Hemoglobin-Based Oxygen Carriers Promote Systemic Hyperfibrinolysis That is Both Dependent and Independent of Plasmin

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Abstract

Background—Hyperfibrinolysis plays an integral role in the genesis of trauma-induced coagulopathy (TIC). Recent data demonstrates that red blood cell (RBC) lysis promotes fibrinolysis; however, the mechanism is unclear. Hemoglobin-based oxygen carriers (HBOC) have been developed for resuscitation and have been associated with coagulopathy. We hypothesize that replacement of whole blood (WB) using an HBOC results in a coagulopathy due to the presence of free hemoglobin.

Materials and Methods—Whole blood was sampled from healthy donors (n=6). The clotting profile of each citrated sample was evaluated using native thrombelastography (TEG). Serial titrations were performed using both HBOC (Polyheme) and normal saline (NS) (5%, 25%, and 50%) and evaluated both with and without a 75 ng/microliter tissue-plasminogen activator (tPA) challenge. Tranexamic acid (TXA) was added to inhibit plasmin dependent fibrinolysis. Fibrinolysis was measured and recorded as LY30, the percentage of clot lysis at 30 minutes after maximal clot strength. Dilution of WB with NS or HBOC was correlated using LY30 via Spearman Rho coefficients. Groups were also compared using a Friedman test and post-hoc analysis with a Bonferroni adjustment.

Results—TPA provoked fibrinolysis was enhanced by both HBOC (median LY30 at 5%, 25%, 50% titrations: 11%, 21%, 44%; Spearman=0.94; p<0.001) and NS (11%, 28%, 58%; Spearman=0.790; p<0.001). However, HBOC also enhanced fibrinolysis without the addition of

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tPA (1%, 4%, 5%; Spearman=0.735; $p=0.001$) and NS did not (1%, 2%, 1%; $r=0.300$; $p=0.186$). Moreover, addition of TXA did not alter or inhibit this fibrinolysis (WB vs 50% HBOC: 1.8% vs 5.7%, $p=0.04$). There was no significant difference in fibrinolysis of HBOC with or without TXA (50% HBOC vs 50% HBOC +TXA: 5.6% vs 5.7%, $p=0.92$). Additionally, the increased fibrinolysis seen with NS was reversed when TXA was present (WB vs 50% NS: 1.8% vs 1.7%, $p=1.0$).

Conclusion—HBOCs enhance fibrinolysis both with and without addition of tPA; moreover, this mechanism is independent of plasmin as the phenomenon persists in the presence of TXA. Our findings indicate the hemoglobin molecule or its components stimulate fibrinolysis by both tPA-dependent and innate mechanisms.

Keywords

Fibrinolysis; Thromboelastography; TEG; Coagulopathy; Trauma-induced Coagulopathy

Introduction

Vascular patency is maintained through fibrinolysis, the physiologic counterbalance to coagulation.(1) Although descriptions of coagulopathic bleeding in trauma date back to more than 200 years ago(2), our knowledge of how the fibrinolytic system is regulated, and how its dysfunction contributes to the development of refractory bleeding remains incomplete. Starzl et al appreciated the physiologic role of systemic fibrinolysis in the 1960's during liver transplantation, using thrombelastography (TEG). They noted that some patients developed excessive bleeding particularly in the early phase of transplant, and initially recommended antifibrinolytics to prevent systemic fibrinolysis.(3) However, this practice was terminated several years later when they noted an increase in venous thromboembolism (VTE) and mortality.(4) Although relatively uncommon, excessive systemic fibrinolysis (hyperfibrinolysis) is associated with uncontrollable bleeding and increased mortality.(5) Percent clot lysis at 30 minutes (LY30) at the time of admission greater than 15% has been associated with a mortality rate of 70% in some trauma reports. (5-7) Conversely, excessive inhibition of the fibrinolytic system (shutdown) is associated with VTE, multiple organ failure, prolonged ICU stay, and significant morbidity.(8) Clearly, tight regulation of fibrinolysis during hemorrhage is key in providing optimal levels of coagulation and fibrinolysis to control bleeding while maintaining systemic vascular patency.

Hemoglobin-based oxygen carriers (HBOCs) have been developed in an attempt to provide oxygen delivery when blood products are not available.(9) Newer generation agents include purified, polymerized hemoglobin molecules. Several of these products have been tested for human use, however, FDA approval remains elusive due to potential adverse events including coagulopathy, nitric oxide (NO) scavenging leading to vasoconstriction, and myocardial ischemia.(10) These purified solutions provide an excellent tool for the preliminary evaluation of hemoglobin as a mediator of fibrinolytic dysfunction using TEG.

The addition of red blood cell and platelet lysates to healthy volunteer whole blood results in hyperfibrinolysis and fibrinolysis shutdown, respectively.(11) Recent proteomic evaluation

of human trauma patients in hemorrhagic shock has shown elevated plasma levels of hemoglobin and its subunits.(12) Therefore, we hypothesize that hemoglobin may be a mediator of postinjury hyperfibrinolysis.

Materials and Methods

Subjects

Assays were performed on citrated whole blood samples collected from healthy volunteers after obtaining informed consent. No one in the study had a known coagulation disorder, nor were they taking any medications known to affect coagulation or fibrinolysis. Each group contained six male subjects with a median age of 30.

Tissue Plasminogen Activator

Human tissue plasminogen activator (tPA) from Molecular Innovation (Novi, MI) was diluted in 5% bovine serum albumin in phosphate buffered saline and aliquoted with a concentration of 10 micrograms per milliliter. Aliquots were stored in liquid nitrogen and thawed immediately prior to each experiment. The dose of tPA administered equated to a final tPA concentration of 75 nanograms/milliliter in each sample. This concentration has previously been found to be an inflection point for which a citrated native TEG increased fibrinolytic activity compared to a non-tPA-challenged whole blood sample in healthy volunteers.(13)

Hemoglobin-based Oxygen Carrier

The HBOC in this study was Polyheme (Northfield Laboratories, IL), a polymer of hemoglobin molecules.(14) Polyheme was stored at 4° Celsius and accessed from its storage container using a sterile coupler and large bore needle.

Tranexamic Acid

Tranexamic acid (TXA) was used to block the action of plasmin. TXA is a synthetic lysine analog that reversibly binds to plasminogen, preventing its interaction with fibrin and conversion to plasmin.(15) TEG cups (Haemonetics, Niles IL) containing air-dried TXA at a concentration of 75ng/ml were stored at 4°C per manufacturer instruction and used to perform assays as described below.

Thrombelastography

Blood was collected in 3.5 ml citrated blood tubes via venipuncture and assayed at room temperature between 20 minutes and 2 hours after blood draw, as recommended by the manufacturer. Whole blood was mixed with a hemoglobin-based oxygen carrier (Polyheme, Northfield, IL) or normal saline (NS) to reach a total volume of 500 microliter in individual Eppendorf tubes (Hamburg, Germany). The fraction of whole blood replaced with HBOC or NS ranged from 5-50%. Assays were performed with and without the addition of tPA (added within 5 minutes of assay start time). TPA assays were also performed in the presence of tranexamic acid (TXA). Citrated native samples were re-calcified, and thrombelastography assays were performed using the TEG 5000 Thrombelastograph Hemostasis Analyzer

(Haemonetics, Niles IL) per manufacturer instructions. Fibrinolysis was measured using the lysis 30 minutes after MA (LY30, %). These assays were compared to control assays performed in the same manner on whole blood without addition of HBOC or NS.

Statistical Analysis

The TEG value LY30 had a non-normal distribution. Spearman's Rho was used to assess the correlation of LY30 values to the percent dilution of whole blood (0%, 5%, 25%, and 50%). Comparisons of the groups WB, WB with tPA, and WB diluted with HBOC or NS at the 5%, 25% and 50% dilution level were performed with non-parametric tests including the paired Friedman's test with the Bonferroni adjustment for multiple comparisons, Kruskal-Wallis test, and the Wilcoxon test. Significance was set at two-tailed alpha of 0.05 for all statistical tests.

Results

In the presence of tPA fibrinolysis is enhanced in a dose-dependent fashion in both the HBOC and NS groups. Figure 1 compares median LY30 (%) of HBOC to NS at increasing doses. Median LY30 at 5%, 25%, and 50% titrations were: HBOC 11%, 21%, and 44% (Spearman's rho coefficient 0.94; $p<0.001$), NS 11%, 28%, 58% (Spearman's rho coefficient 0.79; $p<0.001$).

When the experiments were repeated at 50% replacement in the presence of TXA, LY30 was significantly reduced in both groups (HBOC 5.7% from 44%; $p<0.001$, and NS 1.8% from 58%; $p=0.001$) as compared to the groups without TXA.

Figure 2 compares the LY30 of 50% HBOC with TXA to 50% NS with TXA. Interestingly, fibrinolysis remained elevated above control in the HBOC group, and above NS even after blockage of tPA-mediated fibrinolysis with TXA (HBOC 5.7% vs NS 1.7%; $p=0.009$), (HBOC 5.7% vs WB 1.8% $p=0.009$). Comparison of WB to NS was not statistically significant (1.7% vs 1.8%, $p=1.0$).

Because of this finding, assays were repeated without tPA. In the absence of tPA a dose-dependent increase in LY30 is present in the HBOC group, but not in the NS group. Figure 3 compares the LY30 of HBOC without tPA to NS without tPA at increasing titrations. Median LY30 at 5%, 25%, and 50% replacement were 1%, 4%, 5% (Spearman's rho coefficient 0.735; $p=0.001$) and 1%, 2%, 1% (Spearman's rho coefficient 0.300; $p=0.186$) for HBOC and NS, respectively.

Viewed from another perspective, 50% replacements with HBOC were compared to evaluate tPA independent effects. Figure 4 compares LY30 of 50% HBOC and 50% HBOC with TXA and showed no significant difference (5.6% vs. 5.7%; $p=0.92$). Both 50% HBOC and 50% HBOC with TXA were elevated compared to WB control (1.3%, $p=0.04$).

Please see Supplementary Table 1 for a list of other TEG variables in each group.

Discussion

Our results indicate that addition of HBOC to whole blood stimulates fibrinolysis. This appears to occur through both tPA-dependent and innate mechanisms. Previous studies have shown that this pattern of increase in tPA-dependent systemic fibrinolysis is similar across different resuscitative fluids including NS, LR, albumin, and others, and this is consistent with our results (Fig 1).(16) While the incidence of hyperfibrinolysis in trauma patients is relatively low on admission (2-6%)(5, 7, 17), it increases to 18% at 1 hour post-admission(18) and detection of systemic hyperfibrinolysis in trauma patients increases by 15% for each liter of pre-hospital fluid that is administered.(5) In these scenarios tPA-dependent hyperfibrinolysis is likely due to hemodilution during resuscitation. Hemodilution in trauma can lead to coagulopathy via several mechanisms including the dilution of anti-fibrinolytic proteins.(19, 20) Normally fibrin clots are protected from fibrinolysis via several mechanisms. Alpha-2 antiplasmin is a serine protease inhibitor that rapidly binds and inactivates plasmin, preventing fibrinolysis.(21) Circulating plasma levels of alpha-2 antiplasmin are high at baseline(22), therefore, blood loss and volume resuscitation can result in a rapid reduction in plasma concentration after trauma. Plasminogen activator inhibitor-1 (PAI-1) is released from alpha granules of activated platelets, resulting in platelet rich clots that are resistant to plasmin-mediated fibrinolysis.(23) In addition to reduction of circulating alpha-2 antiplasmin, hemodilution can lead to thrombocytopenia, thereby reducing the release of PAI-1. Moreover, after shock and tissue injury large endothelial stores of tPA can be released rapidly, overwhelming the PAI-1 system.(24) These effects together lead to systemic hyperfibrinolysis that is primarily mediated by the conversion of plasminogen to plasmin by tPA. TXA is a lysine analog that blocks conversion of plasminogen to plasmin, thereby inhibiting fibrinolysis.(25). In a hyperfibrinolytic system that is caused by the above mechanisms relating to plasma dilution and elevated tPA, the addition of TXA would be expected to completely ameliorate this imbalance by blocking the action of tPA. While this held true for the NS group, there was a persistent elevation in LY30 in the HBOC group (Fig 2), suggesting an innate cause of fibrinolysis separate from dilution.

This idea is supported by our finding of a dose-dependent increase in fibrinolysis without the addition of exogenous tPA for HBOC, but not for NS. (Fig. 3) Moreover, this is not inhibited by TXA (Fig. 4). While this increase is small (5%) compared to that seen in the presence of exogenous tPA (44%) it is important to recognize. An LY30 >3% has been identified as a clinical inflection point in severely injured trauma patients (median Injury Severity Score of 30), above which the need for massive transfusion and the risk of mortality markedly increase.(26) Because this increase occurred without addition of tPA, and also in the presence of TXA, it suggests that HBOC has an innate ability to stimulate fibrinolysis independent of tPA. The mechanism is unclear and could be completely plasmin independent, or could be through binding of HBOC to plasminogen, thereby directly converting it to plasmin or blocking inhibitor binding sites. These results are consistent with previous reports that recognize coagulopathy in studies of HBOCs (27, 28), and it is important to note that fibrinolysis may be a component of this coagulopathy.

There are several limitations of our study. First, as with any in vitro study, our model does not include all the components of the injured patient, most notably the endothelium, and this

may confound our results. Additionally, the HBOC used in this study is not currently FDA approved, although we are not aware of any other currently approved agents. Finally, the precise mechanism of fibrinolysis in this study has not been elucidated. Hemoglobin or one of its polypeptide chains appear likely to mediate the observed fibrinolysis, however, such fibrinolytic potential may involve another constituent of the HBOC solution. Future studies will include TEG analysis of the hemoglobin molecule and its subunits to evaluate their effects on fibrinolysis, as well as identification of potential binding partners within the fibrinolytic system to elucidate a mechanism for our results. By understanding this pathway we hope to identify a specific mediator of life-threatening systemic hyperfibrinolysis and develop therapies that can improve mortality in severely injured patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Comparison of LY30 of HBOC and NS at increasing titrations in whole blood (WB) from 5-50%. 75ng/ml tissue-plasminogen activator (tPA) was added to each sample. Median LY30 at 5%, 25%, and 50% titrations were: HBOC 11%, 21%, and 44% (Spearman's rho coefficient 0.94; $p<0.001$), NS 11%, 28%, 58% (Spearman's rho coefficient 0.79; $p<0.001$). 50% titrations were repeated with the addition of Tranexamic Acid (TXA) (HBOC 5.7% from 44%; $p<0.001$, and NS 1.8% from 58%; $p=0.001$).

Figure 2.

Comparison of median LY30 of WB, 50% HBOC and 50% NS with addition of tPA and TXA (HBOC 5.7% vs NS 1.7%; $p=0.009$, HBOC 5.7% vs WB 1.8%; $p=0.009$, NS 1.7% vs WB 1.8%, $p=1.0$). Please note the difference in scale when compared to Figure 1.

Figure 3.

Comparison of LY30 of HBOC to NS without tPA at 5-50% titrations. Median LY30 at 5%, 25%, and 50% titration were: HBOC 1%, 4%, 5% (Spearman's rho coefficient 0.735; $p=0.001$) and NS 1%, 2%, 1% (Spearman's rho coefficient 0.300; $p=0.186$). Please note the difference in scale when compared to Figure 1.

Figure 4.

Comparison of median LY30 of 50% HBOC and 50% HBOC with TXA (5.6% vs. 5.7%; $p=0.92$). Both 50% HBOC and 50% HBOC with TXA were elevated compared to WB control (5.6%, vs 5.7% vs 1.3%, $p=0.04$). Please note the difference in scale when compared to Figure 1.