Evaluation of a lyophilized platelet-derived hemostatic product

James A. Bynum,[1] Michael A. Meledeo,[1] Grantham C. Peltier,[1] Colby S. McIntosh,[1] Ashley S. Taylor,[1] Robbie K. Montgomery,[1] Kristin M. Reddoch-Cardenas,[1] Todd M. Getz,[2] Michael G. Fitzpatrick,[2] and Andrew P. Cap[1]

BACKGROUND: Current limitations of platelet shelf life to 5 days have led to an increasingly greater demand for hemostatic agents with greater longevity. The objective of this study was to evaluate the function of a lyophilized platelet-derived hemostatic product (thrombosome [TS]) as a potential alternative to fresh platelets.

METHODS: Platelets were collected from whole blood from healthy donors. TSs were reconstituted with water and added to various configurations of reassembled whole blood (platelets, plasma, and RBCs); measures included rotational thromboelastometry (ROTEM), optical aggregometry, mitochondrial function, calibrated automated thrombogram, collagen adhesion under flow (shear flow assay), and flow cytometry.

RESULTS: In ROTEM, no differences were observed between maximum clot formation values for contact pathway activation thromboelastometry tests with TSs or platelet samples. Significantly decreased aggregation was observed in the TSs versus platelets ($p < 0.001$ for all agonists). Flow cytometry measures demonstrated significant decreases in glycoprotein Ib expression and increases in phosphatidylserine expression in the TS group ($p < 0.01$). The calibrated automated thrombogram assay was suggestive (lag time and peak thrombin) that the TSs might have some thrombogenic properties. Measurements of mitochondrial function revealed that TSs had no functional mitochondria.

CONCLUSION: In this study, TSs were shown to have nonfunctional mitochondria. ROTEM measures revealed that the TSs had no impact on clot strength. Likewise, compared to platelets, the TSs displayed minimal aggregation, had significantly more phosphatidylserine (measure of activation status), but had the ability to adhere to a collagen surface under flow conditions and contribute to clot formation and induced greater thrombin generation.

Platelets play a critical role in hemostasis during traumatic injury, and their use, combined with other blood products, is associated with lower transfusion requirements and rates of mortality.[1–3] Trauma-related injuries, often resulting in uncontrolled hemorrhage, remain a leading cause of death in both civilian and military settings.[4] Current limitations of platelet shelf life (5 days at room temperature) have led to an increasing demand for hemostatic agents with greater longevity.[5] Approaches to increasing platelet shelf life have focused on storage conditions and have recently shown promise through the combined use of cold storage and platelet additive solutions.[6–8] Additionally, alternative strategies for preserving platelet function include cryopreservation, platelet activation inhibitors, and pathogen-reduction technology with varying levels of effectiveness.[9–11]

One such strategy involves a novel method of lyophilizing platelets for the purpose of developing a hemostatic product that is readily available for use with minimal preparation in trauma settings where conventional component

ABBREVIATIONS: EXTEM = tissue factor pathway activation thromboelastometry; FIBTEM = functional fibrinogen thromboelastometry; HBS = HEPES buffered saline; INTEM = contact pathway activation thromboelastometry; LHPs = lyophilized human platelets; MCF = maximum clot firmness; PPP = platelet-poor plasma; PRP = platelet-rich plasma; PS = phosphatidylserine; ROTEM = rotational thromboelastometry; TS = thrombosome; WB = whole blood.

From the [1]Coagulation and Blood Research Program, U.S. Army Institute of Surgical Research, Fort Sam Houston, Texas; and the [2]Cellphire Inc., Rockville, Maryland.

Address reprint requests to: James A. Bynum, PhD, Coagulation and Blood Research Program, US Army Institute of Surgical Research, Fort Sam Houston, TX 78234; e-mail: james.bynum.civ@mail.mil

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therapy is unavailable. The development of lyophilized or freeze-dried platelets began over 50 years ago.[12] Earlier studies of lyophilized platelets reveal that the freeze-drying process negatively affects the morphology and function of the platelets.[13–15] Various stabilizing methods have been used recently in an effort to preserve morphology and function throughout the often damaging processing steps of the lyophilization method. For example, lyophilized human platelets (LHPs) (Stasix, Entegrion), uses a stabilization technique that covalently cross-links surface membrane proteins and lipids to protect the platelet-like properties during product processing.[16] Another approach that has shown potential is the thrombosome (TS) product produced by Cellphire Inc. The TS lyophilization process uses trehalose as a stabilizer. Trehalose is a disaccharide found in organisms that are capable of surviving intense dehydration. The stabilizing properties of trehalose are well documented, and it has been shown to be spontaneously internalized by human platelets at 37 °C.[17,18] TSs are designed to be a stabilized, infusible, LHP-derived hemostatic agent. TSs are designed to be stable at room temperature for extended periods, and the lightweight nature of the product lends itself to military and other austere environments.

Recent studies have documented a survival advantage to early blood product–based resuscitation of traumatic hemorrhage.[19,20] The development of an immediately available, effective hemostatic agent could potentially contribute to a significant decrease in early battlefield and civilian trauma mortality. The aim of this study was to evaluate the in vitro hemostatic characteristics of TSs. While it is understood that TSs are not meant to function as a direct platelet substitute, we compared them to freshly isolated platelets in order to document the differences between TSs and fresh platelets. Moreover, we further sought to explore potential mechanisms for observed beneficial properties of TS and identify metrics by which clinical applications could be informed, such as potency and dosing.

METHODS

Sample preparation

Whole blood (WB) was collected from healthy volunteers on the days of data collection in citrate vacutainers (3.2% or 10.9 mM final concentration) according to an approved US Army Institute of Surgical Research Standard Operating Procedure. Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 200-g for 10 min, and platelet-poor plasma (PPP) was produced by centrifugation of whole blood at 2000 × *g* for 20 minutes (twice).

TSs were provided as individual lots of LHP product (n = 4) ready for reconstitution with 1 mL of purified water. Samples were rehydrated, swirled gently over 10 minutes, and assayed within 1 hour to preserve function in a simulation of clinical usage. Sample mass after rehydration with 1 mL of water was approximately 1043 mg, and total volume was approximately 1040 μL.

Reassembled WB was constructed by combining RBCs, PRP, PPP, and TSs in the following configurations: 1) 40% hematocrit (Hct) and 250 platelets/nL, 2) 40% Hct with 82.5 TS/nL, 3) 40% Hct with 250 platelets/nL and 82.5 TS/nL, 4) 25% Hct with 100 platelets/nL and 82.5 TS/nL, and 5) 25% Hct with 100 platelets/nL. Configuration 1 served as the control representation of normal blood, while 2 was the TS-only group, and 3 was the combinatorial group; configuration 4 simulated a hemorrhaging patient's blood makeup, where hematocrit and platelet count are significantly depleted and TS have been transfused, while 5 served as the control hemorrhage patient with no TS. To maintain a simulation of physiologic sampling, the concentration of TS was chosen as approximately 33% of a normal platelet count, representing the higher end of the dosing range tested by Cellphire in previous animal models.[21]

Reassembled whole blood was used in rotational thromboelastometry and collagen-platelet adhesion assays; for light transmission aggregometry, calibrated automated thrombogram, and flow cytometry, PRP with 250 platelet/nL was compared with plasma (PPP isolated from freshly collected WB) containing TS at a concentration of 82.5 per nL.

Platelet/TS aggregation

Aggregation of platelets and TS in plasma was evaluated using an optical aggregometer (Chrono-Log Model 700, Chrono-Log Corporation). Briefly, 500 μL of PRP or TS-containing plasma was incubated for 3 minutes at 37 °C before the addition of 10 μL of platelet agonist: 5 μL ADP + 5 μL HEPES buffered saline (HBS), 5 μL collagen +5 μL HBS, 5 μL thrombin receptor-activating peptide-6 + 5 μL HBS, or the combination of 5 μL collagen +5 μL thrombin receptor-activating peptide (all agonists from Chrono-Log Corporation). Aggregation was observed over 6 minutes with terminal absorbance and area under the curve used as endpoints.

High-resolution respirometry

To evaluate the mitochondrial potential and viability of TS compared to fresh platelets, measurement of mitochondrial respiration was performed in a high-resolution oxygraph (Oxygraph-2 k, Oroboros Instruments) at a constant temperature of 37 °C. Calibration with air-saturated Millipore water was performed daily.[6] Platelets and TS were suspended in the 2-mL glass chamber at a concentration of 200×10^6/mL in a mitochondrial respiration medium (MiR05) containing sucrose 110 mM, HEPES 20 mM, taurine 20 mM, K-lactobionate 60 mM, $MgCl_2$ 3 mM, KH_2PO_4 10 mM, ethyleneglycotetraacetic acid 0.5 mM, bovine serum albumin 1 g/L, pH 7.1. Data were collected using software displaying real-time oxygen concentration and oxygen flux, that is, the

negative time derivative of oxygen concentration (DatLab software 4.3, Oroboros Instruments).

Respiration was first allowed to stabilize without any additions at a routine state, that is, in the physiologic coupling state controlled by cellular energy demands on oxidative phosphorylation. Then the ATP synthase inhibitor oligomycin was added to reveal respiration independent of ADP phosphorylation (oligomycin-induced state 4, henceforth denoted as state 4). To evaluate maximal capacity of the electron transfer system, the protonophore carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone was titrated until no further increase in respiration was detected. The electron transfer system was then inhibited by adding rotenone (Complex I inhibitor) and antimycin-A (Complex III inhibitor). The remaining, primarily nonmitochondrial oxygen consumption (residual) was subtracted from the different respiratory states in further analyses. In intact cells, to determine the relative contribution of the different respiratory states, a control ratio was calculated as the ratio of maximal carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone–stimulated respiration and state 4 respiration.

Coagulation function

Coagulation function of reassembled WB samples was evaluated by rotational thromboelastometry (ROTEM Delta; Instrumentation Laboratory) using three tests performed in duplicate according to the manufacturer's instructions: tissue factor pathway activation (EXTEM), contact pathway activation (INTEM), and functional fibrinogen (FIBTEM). ROTEM demonstrates global hemostasis function and allows for comparison of platelet-induced (or TS-induced) changes versus enzymatic or substrate-based changes. EXTEM reagents provide the standard tissue factor activation, best approximating clot formation following wounding, while INTEM reagents offer a picture of contact activation that is important when a new surface area (i.e., TS) is added to the milieu. The FIBTEM assay inactivates platelets through dosing with cytochalasin D and gives the clotting response due to fibrinogen. However, in this particular case, it also allowed us to distinguish healthy platelet function from TS function (since they would seemingly not be inactivated by cytochalasin D).

Thrombin generation

Thrombin generation was measured in PRP and TS-containing plasma without activating reagent in the calibrated automated thrombogram assay by mixing 80 μL of sample with 20 μL of HBS, incubating for 10 minutes at 37 °C, and mixing by autoinjector with 20 μL of FluCa buffer (Z-Gly-Gly-Arg-AMC fluorogenic substrate and calcium chloride, Diagnostica Stago). The absence of activating reagent allows for the inherent thrombogenicity of TS to be evaluated; because tissue factor and phospholipid will strongly activate the reaction, differences between platelet- and thrombosome-induced thrombin generation might be difficult to detect otherwise. In essence, TS and platelets are the activation reagents. Thrombograms were collected in triplicate and compared against thrombin calibrator samples with a microplate fluorometer (Fluorskan Ascent FL, ThermoFisher Scientific) and computer software (Thrombinoscope, Thrombinoscope BV).

Adhesion of platelets/TS to collagen under flow conditions

Adhesion to collagen by platelets or TS was measured using a shear flow assay system (BioFlux 1000, Fluxion Biosciences). Channels of a BioFlux 48-well plate were coated with reagent grade type I collagen (25 μL of a 100 μg/mL stock solution, Helena Laboratories) for 1 hour, followed by a 15-minute wash with 0.5% bovine serum albumin (Sigma-Aldrich); unadhered collagen was flushed out with excess phosphate-buffered saline.

Platelet- and TS-containing plasma samples were mixed with 1 μM of the live/dead stain calcein-AM (ThermoFisher Scientific) and allowed to incubate in the dark at 37 °C for 30 minutes. RBCs were added, with platelet and TS counts adjusted to desired sample concentrations as described previously. Reassembled WB was added to the inlet well and exposed to pneumatic pressure adjusted by the BioFlux control software to generate a shear rate of 920 s^{-1} (e.g., arterial flow) or 4000 s^{-1} to simulate a pathologic flow scenario (e.g., stenotic vessel). To prevent clot formation within the channel and allow for continued observation of platelet adhesion throughout the duration, samples were not recalcified. Bright-field and fluorescent microscopy (490/525 Ex/Em) images of cells or particles adhered to the surface were collected every 30 seconds for a period of 10 minutes using computer software (MetaMorph, Molecular Devices). Endpoints included fluorescence intensity integrated over the viewing area, percentage of area coverage, and rate of accumulation.

Flow cytometry

TS in plasma were examined for platelet markers on a flow cytometer (FACS Canto II, BD Biosciences) equipped with a forward scatter photomultiplier tube for detection of particles smaller than 1 μm. Samples were diluted 10-fold in Hank's Balanced Salt Solution and labeled with fluorescently tagged antibodies for CD41a, CD62P, and CD42b (antibodies from BD Biosciences), and lactadherin (Haematologic Technologies, Inc.) to bind phosphatidylserine (PS). Size gates were set using standard-sized silica beads (Polysciences). Gates were set to identify platelets and TS by size, and events gated between the size range of 100 and 1000 nm were designated as microparticles.

Statistical analysis

Data collection, aggregation, and statistical analyses were performed with computer software (Excel 2010, Microsoft

Corporation; and Prism 7.01, GraphPad Software, Inc.). Two-way analysis of variance tests were used to determine intergroup differences of results obtained with reassembled WB configurations, and unpaired Student's t tests were used to directly compare platelets and TS in assays using them.

RESULTS

Agonist-induced aggregation

Agonist-induced aggregation of platelets was significantly greater than that observed in TS for all agonists. TS responded most significantly to collagen alone as an agonist (10% aggregation), but this was small compared to the response of platelets (60% aggregation; $p < 0.001$; Fig. 1).

High-resolution respirometry

Mitochondrial respiration via the electron transport chain was shown to be negligible in TS; both routine respiration and oxidative burst capacity was near baseline, established by calibration with buffer only control (Fig. 2).

Rotational thromboelastometry

Within each assay conducted with reassembled WB on the ROTEM (INTEM, EXTEM, or FIBTEM), significant differences were not observed between clot times or angles (a measure of clot rate; Fig. 3). There were statistically significant differences observed in lysis indices at 30 minutes (LI30) for the FIBTEM assay when comparing the platelet-only controls or TS-only to platelet + TS combination samples.

The largest differences for ROTEM analysis were observed in clot strength through the maximum clot firmness (MCF) measurement. With INTEM, large differences were observed between platelet-only and TS-only samples (regardless of whether the platelet concentration was 250 or 100 platelets/nL; $p < 0.0001$), platelet-only and platelet +TS samples ($p < .01$), platelet + TS and TS-only samples ($p < 0.0001$), and the simulated hemorrhage sample compared with TS-only samples ($p < 0.01$). With EXTEM, similar results were observed, and there were also significant differences between platelet-only and platelet + TS samples ($p < 0.01$). No differences were observed in FIBTEM, as both platelets and TS had no observable effect due to the introduction of cytochalasin D as a platelet inhibitor. Notably, the INTEM, EXTEM, and FIBTEM activated TS-only samples all had statistically similar MCF values, indicating that TSs were providing no support for clot strength by MCF measurement.

Thrombin generation

PRP and TS-containing plasma demonstrated no observable differences in endogenous thrombin potential, as this parameter more reliably describes the availability of prothrombin and activating enzymes in the plasma. TS demonstrated a significant reduction in lag time of thrombin generation and an increased thrombin peak compared to platelets ($p < 0.05$ for each comparison; Fig. 4).

Flow cytometry

TSs in flow cytometry analysis were shown to have approximately twice as many events categorized as microparticles up to 1000 nm versus those in the size range of platelets (1000–3000 nm), and these microparticles (100–1000 nm) did not express platelet surface markers (Fig. 5). TSs themselves highly expressed CD41a (92.3%) and PS (97.3%) with a lesser but noteworthy expression of CD42b (45.0%) and CD62P (25.5%).

Adhesion to collagen under flow

When subjected to shear flow in a microfluidics channel coated in collagen, calcein-AM–labeled platelets will bind and fluoresce over a period of 10 minutes (Fig. 6A). Reassembled WB with 40% Hct and 250 platelets/nL demonstrates the most significant degree of platelet adhesion, while the addition of labeled TSs to the platelets (40% Hct, 250 platelets/nL, 82.5 TS/nL) resulted in a reduced adherent platelet population. The simulated hemorrhage (25% Hct, 100 platelets/nL) and hemorrhage with TS (25% Hct, 100 platelets/nL, 82.5 TS/nL) samples had negligible

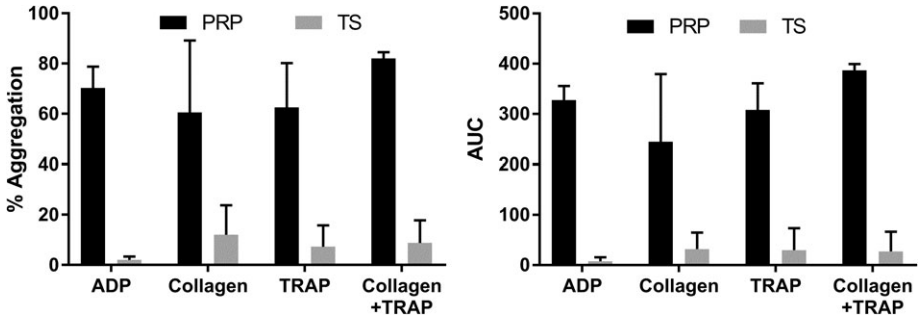

Fig. 1. Aggregation of platelets/TS following agonist stimulation. Responses with TS were significantly for review only smaller than PRP ($p < 0.001$ for all agonists; $n = 4$). Amplitude of aggregation response and area under the curve are shown.**

adhesion profiles due to being below the 30% Hct threshold for effective platelet margination.

While adherent platelets are easily visualized with fluorescence microscopy after labeling with calcein-AM and adhering to collagen (Fig. 6B), reassembled WB with TS and no platelets had no fluorescence profile in the microscopy images, and the use of calcein-AM stain actively inhibited TS binding to collagen as visualized with bright-field imaging (Fig. 6C). Upon further examination with bright-field imaging, unlabeled TSs can be observed in great numbers adhering to collagen, but this adhesion cannot be quantified by the same method because they do not produce calcein-AM fluorescence (Fig. 6D).

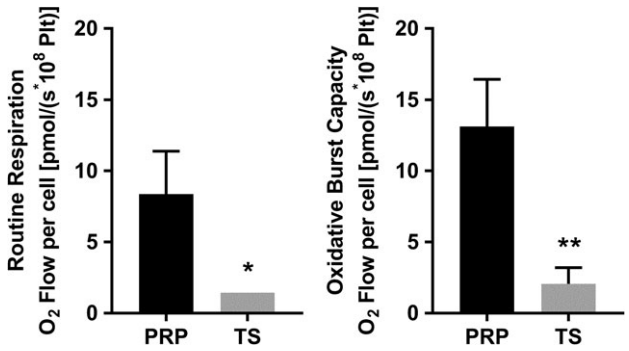

Fig. 2. Mitochondrial function. The respiration function of TS was shown to be negligible and significantly less than PRP (*p < 0.05; n = 4). Both routine respiration and oxidative burst capacity were near baseline, indicating that the mitochondrial function had ceased.

DISCUSSION

For more than half a century, concerted efforts have been applied to the process of preserving platelets through freeze-drying.[12] Attempts to establish efficacy in a small number of thrombocytopenic patients showed promise, but subsequent studies failed to confirm hemostatic efficacy of LHPs.[13,22–24] Earlier failures were attributed to inadequate preservation of platelet integrity during processing. Later attempts to address this issue utilized novel means of cross-linking and additives that demonstrated improved stability, but resulted in significant loss of function.[25,26] More recent LHP products have shown promise primarily through the use of novel stabilizers such as paraformaldehyde and trehalose.[27,28] While this body of work suggests that freeze-dried platelets may retain some metabolic activity that is capable of initiating stimulus-mediated signaling rather than simply providing a procoagulant scaffold for thrombin generation and clot formation, the true nature of these observed properties remains unknown. The objective of the current study was to evaluate the coagulation characteristics of TS in vitro. Furthermore, this study sought to explore the mechanisms of the observed properties of the product and

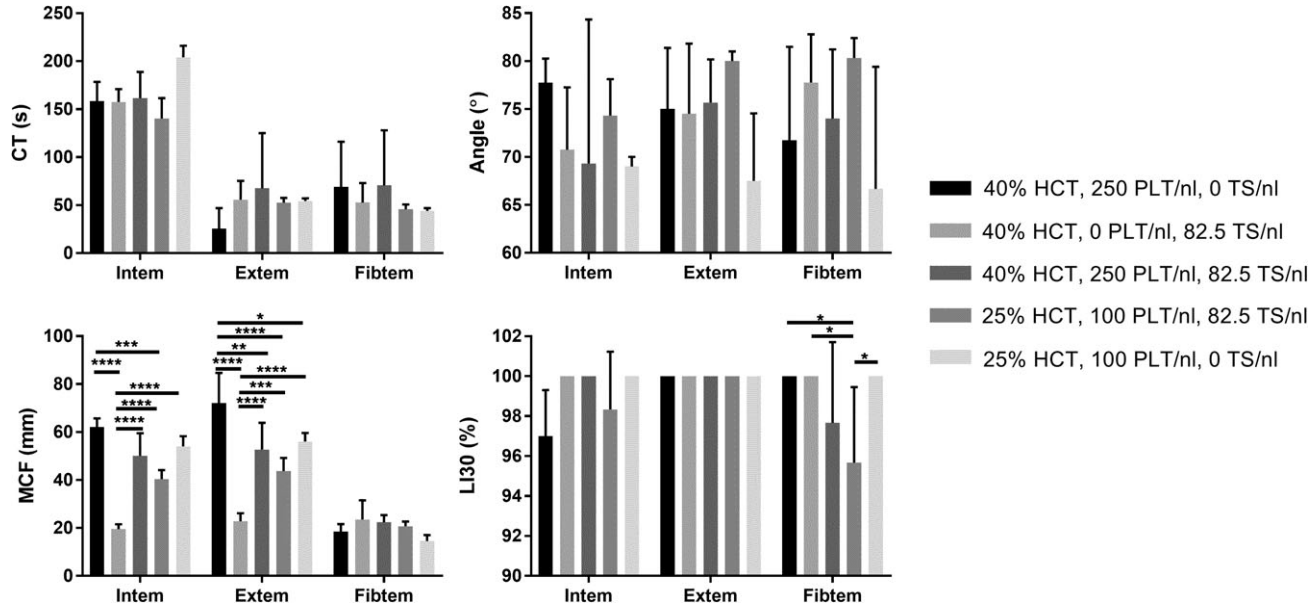

Fig. 3. Coagulation parameters. With INTEM, strong differences were observed between platelet-only and TS-only samples (*p < 0.0001), platelet-only and platelet + TS samples (**p < 0.01), platelet + TS and TS-only samples (***p < 0.0001), and hemorrhage-simulation and TS-only samples (**p < 0.01). With EXTEM, similar results were observed, and there was also a significant difference between platelet-only and platelet + TS samples (**p < 0.01). No differences were observed in FIBTEM, as both platelets and TS had no observable effect. Notably, the INTEM, EXTEM, and FIBTEM activated TS-only samples all had the same MCF, indicating that TS had no impact on clot strength.**

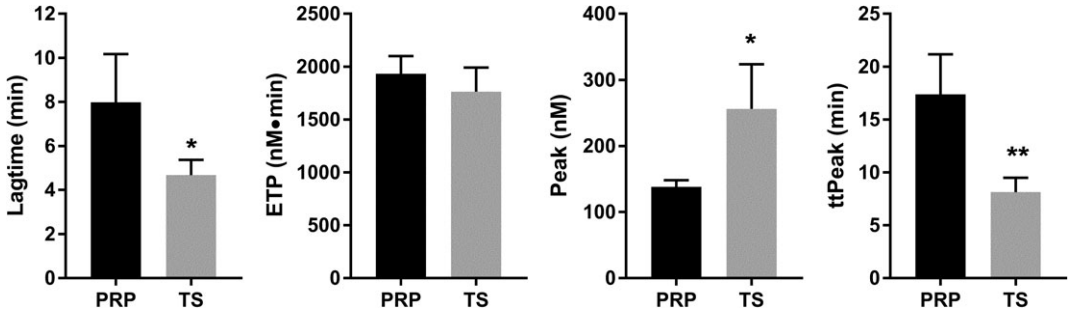

Fig. 4. Calibrated automated thrombogram: measurement of thrombin generation. Peak, time-to-peak, and lag time were significantly different between platelet- and TS-containing samples (*p < 0.05, **p < 0.01; n = 4).

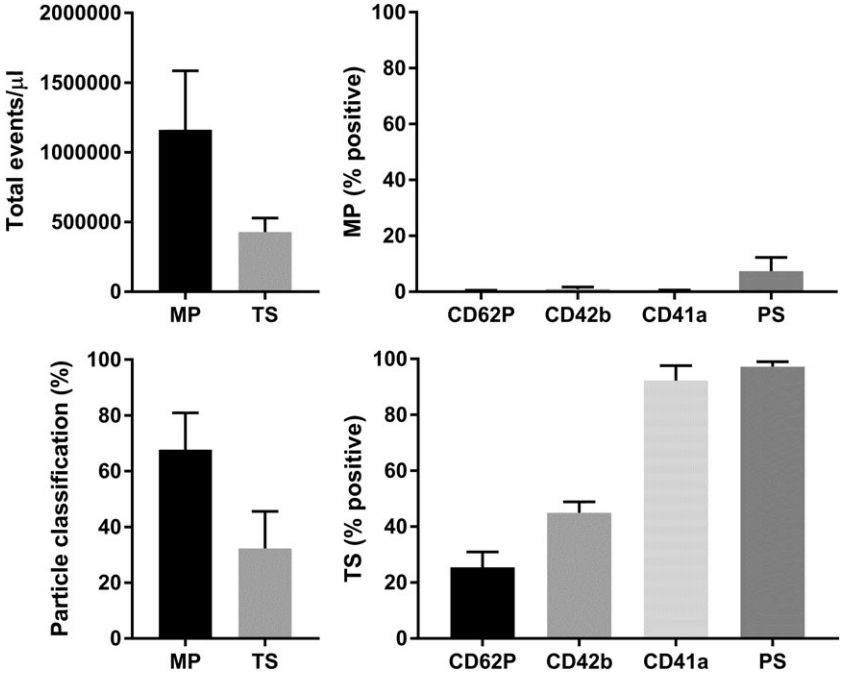

Fig. 5. Flow cytometry: analysis of surface expression of TS. There were roughly twice as many microparticle events versus TS events, but almost none of the microparticles had any expression of platelet markers, indicating that they were likely not of platelet origin. TS, on the other hand, had expression of all four markers, although only PS and CD41a were above 90%.

provide insights into potential potency and dosing guidelines for clinical use.

Paraformaldehyde-fixed LHPs have been shown to be morphologically similar to fresh platelets but demonstrated reductions in glycoprotein Ib-IX, glycoprotein IV, and integrin αIIbβ3.[29] Additionally, platelets preserved by this method showed significant lack of aggregation ability and were not able to express P-selectin or generate thromboxane.[30] However, other studies have shown that they adhere to fibrinogen and generate thrombin.[15]

Previous studies with TS demonstrated that they persist in circulation for up to 24 hours after infusion and were nonimmunogenic in a New Zealand white rabbit model.[16] Additional studies in a thrombocytopenic rabbit ear bleed injury model showed a reduction in blood loss of up to 89.5% compared to control animals.[21,31,32] Furthermore, studies have shown that TS can incorporate into a thrombus and contribute to clot formation.[33] in vitro studies, while limited, have shown similarities in surface marker profiles, some thromboelastogram values, and size distributions to those of stored platelets.[21] Additionally, studies of TS have shown that cellular integrity is greatly preserved compared to previous methods of platelet lyophilization, and TSs retain many of the critical features of fresh platelets including cytoskeletal structures and subcellular organelles. While other LHPs have resulted in thrombotic complications, safety studies in TSs have been favorable in several animal models including baboons, rabbits, and dogs.[31,32] To our knowledge, this study is the first to provide a detailed in vitro evaluation of TSs on hemostasis and metabolic function.

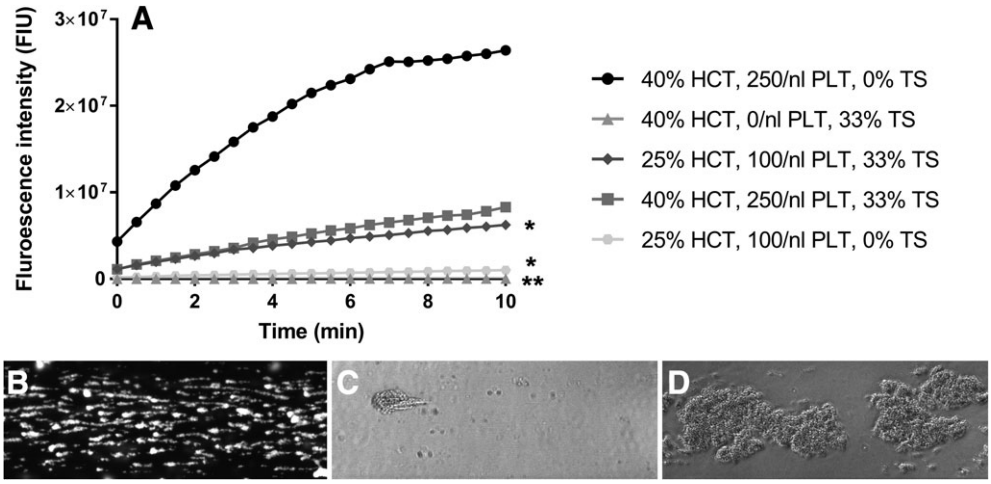

Fig. 6. Adhesion of platelets/TS to collagen surface under shear (920 s-1). (A) Platelets and TSs were labeled with calcein-AM, combined with RBCs and plasma to create five sample conditions. After 10 minutes of flow, significant differences in fluorescence intensity are observed between normal WB 40% Hct, 250 platelets/nL and samples that substituted TSs for platelets at only 33% of the concentration (p < 0.01) or which had depleted platelet count with or without TS addition (*p < 0.05 for each). Samples containing normal platelet count with additional TS added had diminished but not statistically significant differences in fluorescence (p = .068). (B) Calcein-AM stained platelets are easily identified, but no fluorescence could be seen in TS-only samples (not shown). (C) Bright-field images revealed that TSs do not adhere to collagen when stained with calcein-AM. (D) Unlabeled TSs adhere to collagen in large numbers, which cannot be quantified by the same method.**

This work demonstrated that TSs do not aggregate significantly in response to platelet agonists. Since it is known that platelet mitochondria play a critical role in platelet aggregation and secretion by providing the energy needed for these processes,[34,35] we tested the TSs for mitochondrial viability and found that they do not contain functional mitochondria. This is likely a direct result of the lyophilization process, and as such, limits the degree to which TSs can substitute for lost platelet function in a patient.

TS contribution to clot formation was determined by ROTEM and showed similarities to fresh platelets in most measures including reflecting clot inititation and propagation including clot time and angle. However, MCF was significantly decreased in TS-containing mixtures compared to fresh platelets at either normal (250 platelets/nL) or low (100 platelets/nL) concentrations, likely a consequence of the lack of mitochondrial-driven aggregation and clot retraction in TSs, both highly bioenergetic processes.[36] Overall, simulated whole blood containing TS alone provided no increase in clot strength over plasma alone (EXTEM or INTEM vs. FIBTEM comparison; Fig. 3).

As has been demonstrated in other LHP products, TSs generated high peak thrombin as measured by calibrated automated thrombogram. Much of the hemostatic potential of LHPs can be attributed to thrombin generation, which is possibly a result of platelet activation caused during manufacturing, activation that is reflected in the significantly higher expression of PS in the TS population compared to fresh platelets The significant quantity of microparticles present in the TS product may also contribute to thrombin generation; although PS expression was limited in this population, future studies should further characterize these microparticles. Overall, these findings suggest that a significant component of the hemostatic potency of TS may be driven by the high amount of PS expressed on the activated TS surface.

To simulate TS infusion into a vascular injury under flow conditions, we used a collagen-coated surface under arterial and pathologically high shear. TSs could not take up the calcein-AM dye and therefore appeared initially to be incapable of binding collagen under shear. Further examination of the BioFlux plates under bright-field microscopy revealed significant TS binding, but this was impossible to quantify precisely. We conclude that TSs can bind to a simulated injury site exposing collagen and contribute to clot formation under flow conditions. Our study showed that when infused with fresh platelets, labeled TSs inhibited binding of platelets. This result suggests that either TSs may compete for binding sites on collagen when other functional platelets are present or that this apparent competition is an artifact of TS labeling since unlabeled TSs clearly bind to collagen in abundance. Furthermore, given that TSs express a high level of PS on their surface, it is challenging to interpret these data because the assay is performed in a citrated environment where thrombin production is blocked. Thrombin is important not only for the conversion of fibrinogen to fibrin but also for the recruitment of other platelets to sites of injury. Taken together, these data suggest that the potential hemostatic

mechanism of TS may be mediated by collagen binding and local thrombin generation at the sites of injury. Finally, while the results presented herein demonstrate how TS may improve hemostatic potential in simulated clinical conditions; functional in vivo clinical studies will be required to evaluate their actual clinical effect in bleeding patients.

CONCLUSION

This work provides the first detailed evaluation of the in vitro hemostatic and metabolic properties of TSs. We demonstrated that TSs do not possess viable mitochondria, which likely explains the loss of aggregation function of the product and inability to contribute to clot strength as observed by ROTEM MCF. TSs are capable of catalyzing thrombin generation as a result of high PS expression. TSs were shown to adhere to collagen under flow conditions, a critical property of a hemostatic agent meant to stop bleeding. The data presented here suggest the clinical utility of TSs, but further research is required to develop a potency metric for TSs and other platelet-derived hemostatic products that adequately integrates both thrombin generation potential and the ability to localize this to a site of injury through collagen binding. Potency metric development should be validated in clinical studies.

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CONFLICT OF INTEREST

JAB, MAM, GCP, CSM, AST, RKM, KMRC, and APC have disclosed no conflicts of interest. MGF and TMG, who contributed to the manuscript and work, were employees of Cellphire Inc., which manufactured the thrombosomes studied in this work.

REFERENCES

1. Borgman MA, Spinella PC, Holcomb JB, et al. The effect of FFP:RBC ratio on morbidity and mortality in trauma patients based on transfusion prediction score. Vox Sang 2011;101: 44-54.
2. Borgman MA, Spinella PC, Perkins JG, et al. The ratio of blood products transfused affects mortality in patients receiving massive transfusions at a combat support hospital. J Trauma 2007;63:805-13.
3. Spinella PC, Holcomb JB. Resuscitation and transfusion principles for traumatic hemorrhagic shock. Blood Rev 2009;23:231-40.
4. Holcomb JB, Jenkins D, Rhee P, et al. Damage control resuscitation: directly addressing the early coagulopathy of trauma. J Trauma 2007;62:307-10.
5. Hess JR. Conventional blood banking and blood component storage regulation: opportunities for improvement. Blood Transfus 2010;8(Suppl 3):s9-15.
6. Bynum JA, Meledeo MA, Getz TM, et al. Bioenergetic profiling of platelet mitochondria during storage: 4°C storage extends platelet mitochondrial function and viability. Transfusion 2016; 56(Suppl 1):S76-84.
7. Getz TM, Montgomery RK, Bynum JA, et al. Storage of platelets at 4°C in platelet additive solutions prevents aggregate formation and preserves platelet functional responses. Transfusion 2016;56:1320-8.
8. Slichter SJ, Corson J, Jones MK, et al. Exploratory studies of extended storage of apheresis platelets in a platelet additive solution (PAS). Blood 2014;123:271-80.
9. Johnson L, Tan S, Wood B, et al. Refrigeration and cryopreservation of platelets differentially affect platelet metabolism and function: a comparison with conventional platelet storage conditions. Transfusion 2016;56:1807-18.
10. Pidcoke HF, McFaul SJ, Ramasubramanian AK, et al. Primary hemostatic capacity of whole blood: a comprehensive analysis of pathogen reduction and refrigeration effects over time. Transfusion 2013;53(Suppl 1):137S-49S.
11. Bode AP, Holme S, Heaton WA, et al. Extended storage of platelets in an artificial medium with the platelet activation inhibitors prostaglandin E1 and theophylline. Vox Sang 1991; 60:105-12.
12. Bode AP, Fischer TH. Lyophilized platelets: fifty years in the making. Artif Cells Blood Substit Immobil Biotechnol 2007;35: 125-33.
13. Arnold P, Djerassi I, Farber S, et al. The preparation and clinical administration of lyophilized platelet material to children with acute leukemia and aplastic anemia. J Pediatr 1956;49: 517-22.
14. Jackson DP, Sorensen DK, Cronkite EP, et al. Effectiveness of transfusions of fresh and lyophilized platelets in controlling bleeding due to thrombocytopenia. J Clin Invest 1959;38: 1689-97.
15. Brinkhous KM, Read MS. Preservation of platelet receptors for platelet aggregating factor/von Willebrand factor by air drying, freezing, or lyophilization: new stable platelet preparations for von Willebrand factor assays. Thromb Res 1978;13:591-7.
16. Burdette AJ, Andrew Pratt G 3rd, Campagna MV, et al. Evaluation of a new generation platelet-derived hemostatic agent in a rabbit thrombocytopenic model. Thromb Res 2017;158:79-82.
17. Wolkers WF, Looper SA, Fontanilla RA, et al. Temperature dependence of fluid phase endocytosis coincides with membrane properties of pig platelets. Biochim Biophys Acta 2003; 1612:154-63.

18. Wolkers WF, Walker NJ, Tablin F, et al. Human platelets loaded with trehalose survive freeze-drying. Cryobiology 2001;42: 79-87.
19. Howard JT, Kotwal RS, Santos-Lazada AR, et al. Reexamination of a battlefield trauma golden hour policy. J Trauma Acute Care Surg 2018;84:11-8.
20. Shackelford SA, Del Junco DJ, Powell-Dunford N, et al. Association of prehospital blood product transfusion during medical evacuation of combat casualties in Afghanistan with acute and 30-day survival. JAMA 2017;318:1581-91.
21. Fitzpatrick GM, Cliff R, Tandon N. Thrombosomes: a platelet-derived hemostatic agent for control of noncompressible hemorrhage. Transfusion 2013;53(Suppl 1):100S-6S.
22. Klein E, Farber S, Freman G, editors. Effects of dried fraction from platelets on Ca clotting time of normal plasmas and plasma in thrombocytopenia and hemophilia. Fed Proc; 1955.
23. Klein E, Farber S, Djerassi I, et al. Hemostasis following the administration of lyophilized platelet material in secondary thrombocytopenia. Proceedings of the 6th Con-gress of the International Society of Hematology; 1956. p. 525.
24. Klein E, Farber S, Djerassi I, et al. The preparation and clinical administration of lyophilized platelet material to children with acute leukemia and aplastic anemia. J Pediatr 1956;49:517-22.
25. Fischer TH, Merricks EP, Bode AP, et al. Thrombus formation with rehydrated, lyophilized platelets. Hematology 2002;7: 359-69.
26. Bode AP, Read MS. Lyophilized platelets: continued development. Transfus Sci 2000;22:99-105.
27. Bode AP, Read MS, Reddick RL. Activation and adherence of lyophilized human platelets on canine vessel strips in the Baumgartner perfusion chamber. J Lab Clin Med 1999;133: 200-11.
28. Hoshi R, Murata S, Matsuo R, et al. Freeze-dried platelets promote hepatocyte proliferation in mice. Cryobiology 2007;55: 255-60.
29. Ramsey R, Evatt BL. Rapid assay for von Willebrand factor activity using formalin-fixed platelets and microtitration technic. Am J Clin Pathol 1979;72:996-9.
30. Allain JP, Cooper HA, Wagner RH, et al. Platelets fixed with paraformaldehyde: a new reagent for assay of von Willebrand factor and platelet aggregating factor. J Lab Clin Med 1975;85: 318-28.
31. Fitzpatrick GAH, Dee J. Trehalose stabilized freeze dried human platelets, thrombosomes, reduce blood loss in thrombocytopenic rabbit ear bleed model by as much as 89.5%. Vox Sang 2010;99(Suppl 1):0452 (International Society of Blood Transfusion).
32. Fitzpatrick MDJ, Koh A, et al. Characterization of a novel lyophilized platelet derivative (LPDH) which exhibits in vitro hemostatic function, and in vivo safety and efficacy in trauma induced uncontrolled bleeding in nonhuman primate. Blood 2011;118:719 (ASH Annual Meeting Abstracts 2011).
33. Joshi NV, Raftis JB, Lucking AJ, et al. Lyophilised reconstituted human platelets increase thrombus formation in a clinical ex vivo model of deep arterial injury. Thromb Haemost 2012; 108:176-82.
34. Bozza FA, Weyrich AS. Mitochondria push platelets past their prime. Blood 2008;111:2496-7.
35. Zharikov S, Shiva S. Platelet mitochondrial function: from regulation of thrombosis to biomarker of disease. Biochem Soc Trans 2013;41:118-23.
36. Ravi S, Chacko B, Sawada H, et al. Metabolic plasticity in resting and thrombin activated platelets. PLoS One 2015;10: e0123597.