Thrombosomes: a platelet-derived hemostatic agent for control of noncompressible hemorrhage

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BACKGROUND: Uncontrolled hemorrhage is responsible for ~80% of the potentially survivable deaths in combat and over 40% of early mortality in the under 65 age group in the United States. Providing an easily used infusible hemostatic agent to first responders could significantly reduce these fatalities. We report on an infusible lyophilized platelet-derived hemostatic agent stabilized with trehalose and polysucrose prior to and during lyophilization.

STUDY DESIGN AND METHODS: Characterization included determining the particle population size range, surface marker expression GPIb, GPIIbIIIa, and Annexin V binding. Function was assessed by aggregation, thromboelastography, and thrombin generation. Pharmacokinetics, biodistribution, and immunogenicity established using Indium^{111} labeled Thrombosomes in healthy New Zealand white rabbits (NZWRs), efficacy in thrombocytopenic NZWR, and safety in NZWRs, canines, and nonhuman primates.

RESULTS: Thrombosomes retained GPIIbIIIa expression (98.71% ± 0.18 of the rehydrated particles), a reduced expression of GPIb (47.77% ± 6.65), and Annexin V binding (86.05% ± 2.65). Aggregation to all agonists except thrombin in buffer (78.15% ± 2.5) was <50%. Thrombin generation and thromboelastography results demonstrated a concentration gradient that was consistent from lot to lot. There were no observed adverse events in any safety study and blood loss was reduced by >80% in the thrombocytopenic ear bleed model.

CONCLUSION: Our in vitro characterization studies in conjunction with preclinical animal safety and efficacy studies demonstrated lot consistency in manufacturing, maintenance of hemostatic functions of Thrombosomes, safety at high dose concentrations, and the potential to provide an effective hemostatic agent at the site of injury.

One of the missing therapeutics for remote damage control resuscitation (DCR) is an infusible hemostatic agent for the treatment of noncompressible hemorrhage. Cellphire has developed a damage control therapeutic agent, Thrombosomes. This freeze-dried platelet-derived hemostatic agent will provide the combat medic and civilian first responder an easy-to-use effective treatment to reduce blood loss from noncompressible hemorrhage. Thrombosomes are not a platelet substitute but conserve the essential characteristics of platelet function needed to establish a clot at the bleeding site. Thrombosomes adhere to the subendothelial collagenous matrix, rapidly assemble tenase complex, produce thrombin, and convert fibrinogen to fibrin use to form a stable clot.[1] In a modification of the "Blajchman" thrombocytopenic rabbit ear model, Thrombosomes infused 15 minutes before the ear injury resulted in >85% reduction in blood loss.[2] Safety and toxicity studies resulted in no observable adverse events in healthy New Zealand white rabbits (NZWRs), canines, and nonhuman primates (NHPs) with a severe liver injury.

Uncontrolled bleeding is considered one of the highest causes of preventable death in those less than 65 years of age.[3-5] The analysis of the medical records from battlefield casualties indicates that 62% of the potentially preventable deaths that occurred after arrival at a hospital were from hemorrhage.[6] Eastridge and colleagues reviewed the medical records of combat fatalities determining that 83% were due to traumatic brain injury and 16% due to hemorrhage; 80% of the hemorrhage fatalities

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were potentially survivable if the hemorrhage could have been controlled.[7] The military data also demonstrated a survival advantage with the early utilization of platelets and/or plasma at a 1:1 ratio with red blood cells (RBCs), prompting current recommendations to transfuse a single unit of apheresis platelets for every six units of RBCs and plasma (1:1:1 ratio).[8,9]

The Department of Defense, the Armed Services Blood Program Office, and their predecessors have focused efforts on improving blood availability in times of war. Platelet availability is still severely limited in military situations and at rural hospitals. Platelets cannot be routinely provided to military surgeons until the establishment of a safe zone for operations. The primary source of platelets is fresh whole blood, which must be transfused without infectious disease testing. Plateletpheresis instruments have been placed in Iraq and Afghanistan, but those products are also transfused before infectious disease testing. The available US platelet inventory fluctuates due to regional and seasonal shortages and a significant shortage is predicted if a viral pandemic were to occur. A frozen platelet, cryopreserved by the addition of dimethyl sulfoxide developed by Valeri and colleagues at the Naval Blood Research Laboratory,[10,11] is being used by the Netherlands,[12] explored by the Australian Defence Forces,[13] and is in a Phase I clinical trial sponsored by the US Army Medical Materiel Development Agency (personal communication, 2012). While frozen platelets have expanded availability, the –80°C storage requirement and need to transport with dry ice limits their use to a hospital environment.

Thrombosomes have been developed to overcome those limitations and further enhance the ability to treat and reduce noncompressible hemorrhage at the site of injury in emergent or battle situations. Thrombosomes are prepared from a pool (5-10) of Group O in-date leukoreduced apheresis platelet units. They are provided in freeze-dried form, can be prepared for use in 5-10 minutes by the addition of sterile water, and have demonstrated conservation of essential characteristics required to form a clot, primary adhesion at the site of injury, leading to aggregation, thrombin production, fibrinogen binding, formation of fibrin, and wound closure. The in vitro and in vivo reported studies provide data on the characterization (physical properties), function, safety, and efficacy of Thrombosomes after rehydration.

PHYSICAL CHARACTERIZATION

Multiple lots of Thrombosomes (Table 1) were evaluated for surface marker expression and particle size distribution. The analysis of particle size was performed with the use of a Becton Dickinson fluorescence-activated cell sorter scan flow cytometer (BD Biosciences, San Jose, CA). Particles labeled with fluorescein isothiocyanate (FITC)-mouse anti-human glycoprotein (GP)IIb-IIIa and phycoerythrin (PE)-labeled mouse anti-human P-selectin antibodies were compared with the fluorescence of a dual FITC and/or PE isotype-labeled antibody sample. Particles exhibiting more intense fluorescence than the nonspecific isotype were then plotted on the fluorescence-activated cell-sorting forward scatter axis. Points on this histogram were separated by regions established with the use of the National Institute of Standards and Technology standard sizing beads and represented as the percentage of positively fluorescent particles below 1.03 μm, those between 1.03 and 5.04 μm, and those larger than 5.04 μm (lower limit of resolution established at 0.2 μm and the upper limit of resolution at 30-40 μm). Surface marker expression was established on the same instrument with FITC-labeled probes: mouse isotype IgG, mouse anti-human GPIb IgG, Annexin V (Becton Dickinson), and FITC-mouse anti-human GPIIb-IIIa IgG (Beckman Coulter). Platelets or rehydrated Thrombosomes were diluted to approximately 50,000/μL in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-modified Tyrode's albumin (HMTA-9.5 mM HEPES, 145 mM NaCl, 4.8 mM KCl, 12.0 mM $NaHCO_3$, 5.0 mM glucose, and 0.35% bovine serum albumin pH adjusted to 7.35) and 50 μL of diluted sample was combined with 5 μL of FITC-labeled antibody and placed in the dark at room temperature (RT) for a 30-minute incubation. Test samples used to determine Annexin V binding were supplemented with calcium (2 mM). Following incubation, 2 mL of HEPES buffered saline (10 mM HEPES and 150 mM NaCl pH adjusted 7.35) was added to each sample. Ten thousand events were collected in the forward and/or side scatter region drawn around fresh platelets (Table 1). Rehydrated Thrombosomes exhibited changes in physical characteristics when compared with the 1- to 2-day-old stored platelets (data not shown) used for manufacturing. The percentage of microparticles (<1.03 μm) of 32.45 (standard error of the mean [SEM] 1.76%) was greater than the 9%-16% observed in the 2-day-old starting material. The reduction in percent-

TABLE 1. Physical characterization

		Mean	SEM
Particles/mL × 10^9		2.09	0.07
Percentage of particles in each size range	<1.03 μm	32.45	1.76
	1.03-5.04 μm	67.70	1.59
	>5.04 μm	0.85	0.34
Surface markers % positive	GPIb	47.77	6.65
	IIbIIIa	98.71	0.18
	Annexin V	86.07	2.65
	Annexin V (activated)	89.64	1.96

GP = glycoprotein; SEM, standard error of the mean.

age of particles positive for GPIb 47.7% (SEM 6.65%) versus 97%-99%, and increased Annexin V binding observed in both resting 86.07% (SEM 2.65%) and activated 89.64% (SEM 1.96%) versus 2.6%-5.2% (resting) and 38%-58% (activated) before manufacturing is consistent with published values for activated platelets.[14]

FUNCTIONAL CHARACTERIZATION†

Aggregation studies were performed in an AggRam aggregometer (Helena Laboratories, Beaumont, TX). Thrombosomes were suspended either in George King pooled normal plasma (George King platelet-poor plasma [GK-PPP]) or HMTA. Thrombosomes particles or platelet concentration was adjusted to 250,000/μL and aggregation was initiated by the addition of 1/10th volume of stock agonist: collagen 100 μg/mL, arachidonic acid 5 mg/mL, ristocetin 12 mg/mL, adenosine diphosphate 200 μM, or thrombin (25 U/mL). Thrombin-induced aggregation was examined only in HMTA buffer as the addition of thrombin to plasma results in clot formation.

Thrombin generation was measured with the use of a modification of the Technothrombin thrombin generation assay test kit, a fluorescent kinetic plate-based assay (Technoclone GmbH, Vienna, Austria). Each assay well included 40 μL of Thrombosomes diluted in a filtered citrated plasma sample (GK-PPP), 10 μL of phospholipid reagent, and 50 μL of fluorogenic substrate and/or calcium. Studies were performed with Thrombosomes concentrations ranging from 5000 to 500,000/μL. Tecan Safire^2 fluorescent plate reader (Tecan Group Ltd., Männedorf, Switzerland) was used to record fluorescent signal every minute over 60 minutes. Lag time, peak time, peak rate, and total thrombin generated were calculated.

Clot formation was assessed with the use of the TEG 5000 Thrombelastograph Hemostasis Analyzer System (Braintree, MA). The standard method was modified substituting GK-PPP with 20 k/μL human in-date stored platelet (hIDSP) in HMTA buffer for whole blood or plasma, and varying concentrations of Thrombosomes in HMTA buffer for a total volume of 340 μL. Thrombosomes were rehydrated in sterile water for injection and gently swirled for 5-10 minutes at RT. Once rehydrated, Thrombosomes were diluted in HMTA buffer to an appropriate concentration (50, 100, or 200 k/μL) and were combined with 20 k/μL of hIDSPs in HMTA buffer (for a total of 170 μL). Reaction was initiated by the addition of 170 μL GK-PPP and 20 μL of 0.2 M $CaCl_2$ to each cup.

TABLE 2. In vitro function measures

		Mean	SEM
Thrombin generation @ 500 k/μL	Peak rate (nM/min)	489.44	12.04
	Total thrombin (nM)	2361.21	68.61
Thrombin generation @ 187.5 k/μL	Peak rate (nM/min)	387.26	18.10
	Total thrombin (nM)	1623.51	127.20
Thromboelastograph @ 200 k/μL	R-time (min)	9.47	0.39
	K-time (min)	4.11	0.22
	Angle (degree)	48.29	2.22
	MA (mm)	38.49	1.22
Thromboelastograph @ 100 k/μL	R-time (min)	10.58	0.26
	K-time (min)	4.91	0.28
	Angle (degree)	40.22	1.55
	MA (mm)	37.20	0.78
Aggregation in plasma	No agonist	15.78	1.23
	Collagen	27.23	2.87
	Arachidonic acid	33.72	3.87
Aggregation in buffer	No agonist	9.70	0.81
	Arachidonic acid	47.17	2.59
	Thrombin	78.15	2.50

MA, maximum amplitude; SEM, standard error of the mean.

Results of the functional assays (Table 2) indicated that Thrombosomes demonstrate mechanistic and metabolic functions required for hemostasis. Thrombin generation was tested at three concentrations to determine if a dose response was observed in proportion to an increase in Thrombosomes concentration. Concentrations of 50,000, 187,500, and 500,000 Thrombosomes per microliter were tested. Peak rate and total thrombin generated by samples containing 50,000 particles/μL were not significantly different from the plasma control (data not shown). The peak rate (nM thrombin/min) increased from 387.26 at the 187,500 particles/μL concentration to 489.44 when 500,000 particles/μL were used. This was a significant difference ($p < 0.05$). The rate (α) and strength (maximum amplitude [MA]) of the clot formed as measured by TEG indicated that the addition of Thrombosomes at concentrations of 100,000 or 200,000/μL to platelet-poor plasma (PPP-platelet count 20,000/μL) improved the MA values from 31.9 ± 13.8 (data not shown) to 37.2 (SEM 0.78) and 38.49 (SEM 1.27), respectively. A similar improvement was observed in the rate of clot formation from the GK-PPP mean α of 31.9 ± 0.9 (data not shown) to 40.22 (SEM 1.55) and 48.9 (SEM 2.22) when Thrombosomes particles were added at concentrations of 100,000 or 200,000/μL. Thrombosomes aggregation to all agonists except thrombin in buffer (78.15% SEM 2.5) was significantly reduced when compared with values from the starting hIDSP pool (data not shown).

† All particle concentrations used for functional characterization and animal safety studies were calculated based on the prelyophilization particle count using the Beckman Coulter AcT10 Hematology Analyzer.

BIODISTRIBUTION AND CIRCULATION PERSISTENCE

Human platelet-derived Thrombosomes, labeled with ^{111}In-oxime, were injected in NZWRs. To control for the influence of intraspecies and interspecies difference, rabbit freeze-dried platelets (RFDPs) were also made from autologous and allogeneic donor rabbits. The distribution of ^{111}In-Thrombosomes was followed by a combination of imaging, blood sampling, and biodistribution at necropsy. The results were compared with the observations made after administration of ^{111}In-labeled fresh rabbit platelets. Circulation kinetics and biodistribution of ^{111}In-Thrombosomes and 7-day stored platelets were also investigated in rabbits with a 14-day prior exposure to intravenous (IV) Thrombosomes or 7-day stored human platelets. Presensitization and postsensitization samples (Thrombosomes) were submitted to Blood Center of Wisconsin, Milwaukee, WI for detection of antibodies to human platelet antigens (unpublished observation).

The circulation persistence of Thrombosomes and RFDP was nearly identical at 2 hours. A biphasic pattern of clearance was characterized by rapid and major drop in circulation within the first 10 minutes after administration; however, the magnitude of drop was much higher in the case of freeze-dried products.[15] The circulation kinetics of postsensitization infusion of radiolabeled Thrombosomes or RFDP over 2 hours of monitoring was not significantly different from the initial circulation studies in naïve animals (Fig. 1). However, the circulation kinetics of 7-day stored platelets in rabbits post-IV sensitization with 7-day stored platelets was significantly reduced (over 95% removed from circulation within 10 min), suggesting a rapid clearance of infused

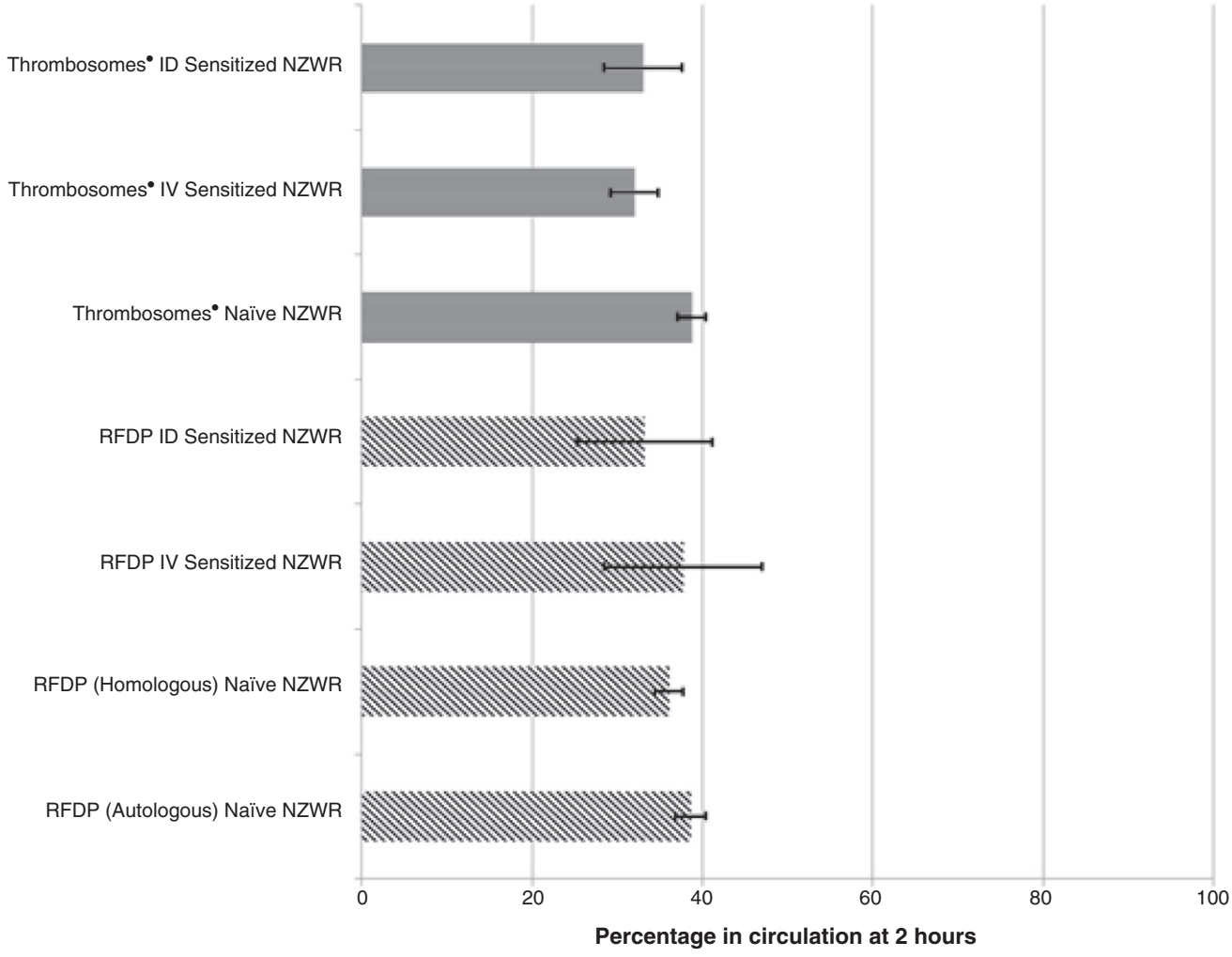

Fig. 1. Circulation persistence studies of Thrombosomes or rabbit freeze-dried platelets (RFDPs) in naïve and intravenous (IV) or intradermal (ID) sensitized New Zealand white rabbit (NZWR). There were no significant differences in circulation persistence in naïve or sensitized animals ($p > 0.05$, $n = 6$ in each group).

7-day-old human radiolabeled platelets in platelet-immunized rabbits, possibly mediated by anti-human platelet antibodies generated from the initial infusion. In Thrombosomes-immunized animals, the circulation persistence of infused radiolabeled Thrombosomes at 10 minutes was greater than 30% and similar to previous studies in naïve animals (Fig. 1). Improved circulation persistence of Thrombosomes and the absence of antibodies to human platelet antigens in response to Thrombosomes sensitization in preinfusion and postinfusion plasmas (data not shown) suggest that priming of rabbits with human Thrombosomes does not result in an immunogenic response that alters the circulation persistence of a second dose in the same animal or results in antibodies directed toward human platelet antigens. The modifications required to stabilize the hIDSP before lyophilization and the lyophilization procedure did not alter the rabbit platelets in a way that formed new epitopes that could have stimulated antibody production and increased the rate of removal, reducing the circulation persistence.

SAFETY AND TOXICOLOGY STUDIES IN HEALTHY NZWRs AND CANINES (BEAGLE)

Healthy NZWRs were infused with a single high dose, the maximum volume of Thrombosomes (70 mL, 1.4×10^{11} particles) or Thrombosomes vehicle control allowable in a single infusion. Animals were monitored and sacrificed 2 and 14 days after infusion. Blood samples were collected periodically for full hematology, coagulation, and clinical chemistry testing. Postmortem study included full macroscopic and microscopic evaluation of organs and tissue.

Healthy canines (beagles) were infused with a high (1.6×10^{10}/kg) or low dose (5.0×10^{9}/kg) of Thrombosomes or an equal volume of Thrombosomes vehicle control. Animals were assessed in the same manner as in the NZWR study with the addition of an expanded coagulation study panel including fibrin degradation products.

NHPS (RHESUS MACAQUE) SAFETY STUDY—CLOSED ABDOMEN HEMORRHAGIC SHOCK IN ANESTHETIZED NHP

Animals (rhesus macaque) were anesthetized, utilizing a laparoscopic technique; a 60% hepatectomy of the left liver lobe was performed initiating time zero. Fifteen minutes later, 50 mL normal saline or Thrombosomes high (2.27×10^{9} particles/kg) or low (2.27×10^{8} particles/kg) dose diluted in 50 mL saline were transfused at approximately 4 mL/min with the use of a syringe pump. Two hours postinjury, an open laparotomy was performed; the shed blood in the abdominal cavity was collected on preweighed gauze sponges that were removed and weighed to determine cumulative blood loss. Blood samples were collected before the initiation of the protocol and periodically thereafter over 8 hours for chemistry, hematology, and coagulation testing.

ANIMAL SAFETY AND TOXICOLOGY STUDY RESULTS

No observable adverse events were reported in high-dose safety studies in healthy NZWRs and canines, and high and low studies in NHP after a liver injury severe enough to induce acute hemorrhagic shock. Evaluation included group pairwise comparisons of clinical laboratory results, and animal and organ weights. Postmortem study included macroscopic and microscopic examination with the use of standard histopathology techniques. No test article-related adverse findings were reported.

DISCUSSION

DCR includes the early and increased use of fresh frozen plasma, platelets, and RBC over crystalloid. There have been numerous publications over the past few years regarding the use of fresh whole blood and "optimized" ratios and times of infusion of platelets, plasma, and RBCs.[16-28] The standard of care regarding transfusion of blood components to trauma patients is receiving national attention. Two major multicenter trauma studies—Prospective, Observational, Multi-center, Massive Transfusion sTudy (PROMMTT) (http://cetir-tmc.org/research/prommtt) and Pragmatic, Randomized, Optimal Platelet and Plasma Ratios (PROPPR) (http://cetir-tmc.org/research/proppr)—were initiated to gather data on current practices (PROMMTT) and to develop prospective randomized trials (PROPPR) to determine the impact of the application of different ratios and order of infusion of platelets and plasma to actively bleeding trauma victims.

Freeze-drying techniques have been applied to platelets since the 1950s without success. The key elements required for approval of a new product are the following: 1) evidence of consistent manufacturing and the ability to fully characterize the product's identity, purity, and potency; 2) demonstration of safety and lack of toxicity in appropriate healthy animal models; and 3) evidence of efficacy in a relevant animal model. Thrombosomes manufactured in support of the animal safety and toxicity studies, persistence and circulation studies, immunogenicity, and efficacy have been fully characterized and demonstrate a consistency in manufacturing as evidenced by the SEM of each parameter. There were no article-related adverse events reported from safety and toxicity studies in two healthy species (NZWR and canine) and from a severely compromised hemorrhagic shock NHP model. Circulation and persistence of RFDPs and Thrombosomes in naïve animals were nearly identical, demonstrated a biphasic pattern, and showed consistency

between lots. Subsequent studies in animals sensitized by IV infusion showed no observable change from controls and from the naïve animals. A positive control with the use of 7-day-old stored platelets was employed, the shelf life of platelet concentrates had been extended to 7 days at the time, and Food and Drug Administration (FDA) guidance was to use the 7-day product as a comparator. As expected, the circulation persistence was significantly reduced (greater than 95% of 7-day-old radiolabeled platelets were removed from circulation within 10 min of infusion). However, animals infused with radiolabeled Thrombosomes on Day 14 exhibited no change in circulation kinetic from the naïve animals. Although the circulation persistence of Thrombosomes is short, it does not appear to impact the efficacy of the product in the thrombocytopenic rabbit ear bleed model described earlier.[2]

CONCLUSION

Thrombosomes retain the essential characteristics needed to initiate and amplify hemostasis. Characterization studies not only support the conservation of essential surface markers and pathways from the liquid-stored RT platelets used as starting material but also show the distinct differences emphasizing that Thrombosomes are neither platelets nor a platelet substitute. Hemostatic function is supported by the evidence of adhesion to the injured vascular tissue matrix, assembly of tenase complex on aggregated platelets, and thrombin generation, leading to clot stabilization. Thrombosomes have prolonged stability when stored at ambient temperatures and when reconstituted with sterile water, can be delivered within minutes as an effective IV hemostatic agent that significantly reduces bleeding in an animal model, and has the potential to be a therapeutic agent for use in DCR.

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CONFLICT OF INTEREST

The authors are employees of Cellphire, Inc., Rockville, MD.

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