Development and Testing of Freeze-Dried Plasma for the Treatment of Trauma-Associated Coagulopathy

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Background: Trauma-induced coagulopathy is associated with an extremely high mortality. We have recently shown that survival can be improved by correction of coagulopathy through early, aggressive infusion of Fresh Frozen Plasma (FFP). However, FFP is a perishable product, and its use is impractical in challenging environments such as a battlefield. Development of shelf-stable, easy to use, low volume, lyophilized, Freeze-Dried Plasma (FDP) can overcome the logistical limitations. We hereby report the development and testing of such a product.

Methods: Plasma separated from fresh porcine blood (n = 10) was either stored as FFP, or lyophilized to produce the FDP. For in vitro testing, the FDP was rehydrated with distilled water and the pH, temperature, and osmolarity were adjusted to match the thawed FFP. Laboratory analysis included measurements of prothrombin time (PT), partial thromboplastin time, fibrinogen levels, and clotting factors II, VII, and IX. To test in vivo efficacy, swine were subjected to multiple injuries (femur fracture and grade V liver injury) and severe hemorrhagic shock (60% blood loss associated with "lethal triad" of coagulopathy, acidosis, and hypothermia), and resuscitated with FFP or FDP (n = 6/group; plasma volumes equal to the volume of shed blood). No treatment, and resuscitation with fresh whole blood served as the control groups (n = 6/group). Coagulation profiles (thromboelastography, PT, partial thromboplastin time, international normalized ratio, fibrinogen) were measured serially during the experiment, and for 4 hours posttreatment.

Results: In vitro analysis revealed no differences in the coagulation profiles of FFP and FDP. The lyophilization process did not decrease the activity levels of the measured clotting factors. In the swine model, multiple injuries and hemorrhagic shock caused a 50% to 70% increase in PT ($p = 0.03$), and infusion of FDP and FFP were equally effective in correcting the coagulopathy.

Conclusion: Plasma can be lyophilized and freeze-dried to create a logistically superior product without compromising its hemostatic properties. This product may be suitable for use in austere environments, such as a battlefield, for the treatment of trauma-associated coagulopathy.

Key Words: Lyophilized, Freeze-dried, Plasma, Blood, Coagulopathy, Acidosis, Hypothermia, Multiple injuries, Resuscitation, Hemodilution, Liver injury, Femur fracture.

J Trauma. 2008;65:975–985.

Hemodilution, hypothermia, acidosis, tissue hypoperfusion, and consumption of clotting factors are common reasons for the development of coagulopathy in trauma patients.[1–10] Early coagulopathy is a marker of injury severity, as 25% of trauma patients are found to be coagulopathic (prothrombin time [PT] >18 seconds or partial thromboplastin time [PTT] >60 seconds) on initial presentation,[11] and is associated with significantly greater mortality (46%) compared with trauma patients who are not coagulopathic. Two other retrospective analyses of over 20,000 trauma patients have reported similar findings despite differences in the definitions of coagulopathy and the volume of prehospital resuscitation fluids.[8,12] In addition to mortality, it has also been shown that patients with acute traumatic coagulopathy have longer length of stay (intensive care unit and hospital), fewer ventilator-free days,[10,11] and an increased likelihood to develop acute lung and renal injuries.[10]

Coagulopathy, together with hypothermia and acidosis, forms a "lethal triad" that is associated with poor prognosis.[13] Early and effective reversal of coagulopathy is important but the best method to achieve this goal remains controversial. Transfusion of blood components remains the mainstay of treatment, with some recent studies advocating a more aggressive approach toward fresh frozen plasma (FFP) administration.[14,15] When component therapy is not available, military hospitals often resort to using fresh whole blood (FWB) for this purpose. According to recent reports, 13% of all transfused patients in

Submitted for publication February 1, 2008.

Accepted for publication May 16, 2008.

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Supported by a grant from the Office of Naval Research (to H.B.A.).

Presented at the 38th Annual Meeting of the Western Trauma Association, February 24–March 1, 2008, Squaw Creek, Olympic Valley, California.

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DOI: 10.1097/TA.0b013e3181801cd9

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 NOV 2008	**N/A**	**-**

4. TITLE AND SUBTITLE
Development and testing of freeze dried plasma for the treatment of trauma-associated coagulopathy.

5a. CONTRACT NUMBER
5b. GRANT NUMBER
5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Shuja F., Shults C., Duggan M., Tabbara M., Butt M. U., Fischer T. H., Schreiber M. A., Holcomb J. B., Sondeen J. L., deMoya M., Velmahos G. C., Alam H. B.,

5d. PROJECT NUMBER
5e. TASK NUMBER
5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
United States Army Institute of Surgical Research, JBSA Fort Sam Houston, TX 78234

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**11**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

the Operation Iraqi Freedom received FWB.[16] The use of FWB, FFP, and platelets for the treatment of trauma-associated coagulopathy has some logistic limitations, especially in austere environments such as a battlefield. FWB carries the risk of transmitting infections and requires availability of appropriate donors. Platelets need to be stored at 20°C to 24°C and used within 5 days to 7 days. Frozen plasma needs to be prepared within 8 hours to 24 hours and can only be stored at −18°C for a maximum of 1 year. It also requires refrigerated transportation, 30 minutes to thaw and should ideally be used within 6 hours of thawing.[17] As most of the battlefield deaths take place before reaching a medical facility, there is a clear need for the development of innovative and effective strategies for the early (prehospital) treatment of coagulopathy. One solution is to convert FFP into shelf-stable, lyophilized Freeze-Dried Plasma (FDP). Such a lyophilized product would have a number of advantages over FFP including: storage at ambient temperature, longer shelf life, quicker preparation time, ABO universality, and reliable viral inactivation methods. We hereby report the development of FDP, followed by in vitro evaluation and testing in a clinically relevant large animal multiple injuries model.

MATERIALS AND METHODS

Preparation of Freeze-Dried Plasma

All processing of porcine plasma for the lyophilization was performed under sterile tissue culture conditions. Frozen porcine plasma (stored at −70°C) was thawed, incubated at 23°C for 1 hour, and centrifuged at 3,000*g* for 10 minutes with retention of the resulting supernatant. In preparation for lyophilization, 300 mL of the plasma supernatant was placed in a sterile 1 L cylindrical Pyrex (10 cm diameter, 25 cm height) vessel, and then frozen in an even layer on the bottom and side of the container by slow rotation in isopropanol dry-ice slurry. The resulting frozen plasma was then freeze-dried for 48 hours at −8°C in a Virtus Unitop 600 SL lyophilizer.[18] Much of the CO_2 in FDP is removed during lyophilization, resulting in an alkaline pH (>8.2) upon rehydration. The freeze-dried plasma product was stored at 4°C until day of use, and then rehydrated with the original volume of sterile water (300 mL portions). The rehydrated plasma was titrated with 20 mmol/L glycine (pH = 2.4) to obtain a final pH = 7.4. All the FDP was used within 1 day of reconstitution.

In Vitro Analysis

FFP and reconstituted FDP were analyzed by the core coagulation laboratory at the Massachusetts General Hospital (Boston, MA) for the level of activity of selected clotting factors (II, VII, and IX). PT, PTT, international normalized ratio (INR), and fibrinogen levels were measured using the STart four machine (Diagnostica Stago, Parsippany, NJ).

In Vivo Testing

All the research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals. The study adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council (latest edition), and was approved by the Institutional Animal Care and Use Committee. All of the procedures were performed under the supervision of a veterinarian. This highly reproducible model was initially developed as part of a multicenter study,[19] by teams from Oregon Health and Science University, Portland, OR, the US Army Institute of Surgical Research, San Antonio, TX (funded by the US Army Medical Research and Materiel Command), and Massachusetts General Hospital/Harvard Medical School, Boston, MA (funded by the Office of Naval Research), and included multiple injuries, large volume blood loss, hemodilution, hypothermia, acidosis, and coagulopathy.

Animal Selection and Preparation

Female Yorkshire swine (80–120 lbs; Tufts Veterinary School, MA) were used for the experiment. Food was withheld the night before the procedure but access to water was allowed. Animals were sedated with 8 mg/kg Telazol (tiletamine hydrochloride 50 mg/mL; and zolazepam hydrochloride, 50 mg/mL; Fort Dodge Animal Health, Fort Dodge, IA) along with 1.2 mg of atropine intramuscularly. They were placed in the supine position and anesthesia was induced with ~4% inhaled isoflurane in 100% oxygen. Animals were then intubated with a cuffed silastic endotracheal tube 7.0 mm (i.d) × 55 cm, and mechanical ventilation was started (Narkomed—M, North American Drager, Telford Pennsylvania) with the initial tidal volume and pressure set at 10 mL/kg body weight, peak pressure at 20 cm H_2O, and 10 to 12 breaths per minute to maintain baseline (BL) end-tidal P_{CO_2} of 40 mm Hg ± 2 mm Hg, with occasional sigh breaths. Reflexes and muscle relaxation were used to monitor the depth of anesthesia, and isoflurane dose titrated between 1% and 3%.

Instrumentation

A five French arterial catheter was placed via a cut down technique in the right femoral artery, and used for continuous monitoring of blood pressure. The right internal carotid artery and internal jugular vein were cannulated with nine French introducer sheaths (Arrow International, Reading, PA). The venous sheath was used for fluid or blood product infusion and the arterial sheath was used for blood withdrawal (hemorrhage). The right external jugular was cannulated for the measurement of central venous pressure as well as infusion of calcium chloride during citrated blood product infusion. A midline laparotomy was performed from the xiphoid process to the inferior aspect of the bladder, and the falciform ligament was divided to gain access to the superior aspect of the liver. A cystostomy was placed for measurement of urine

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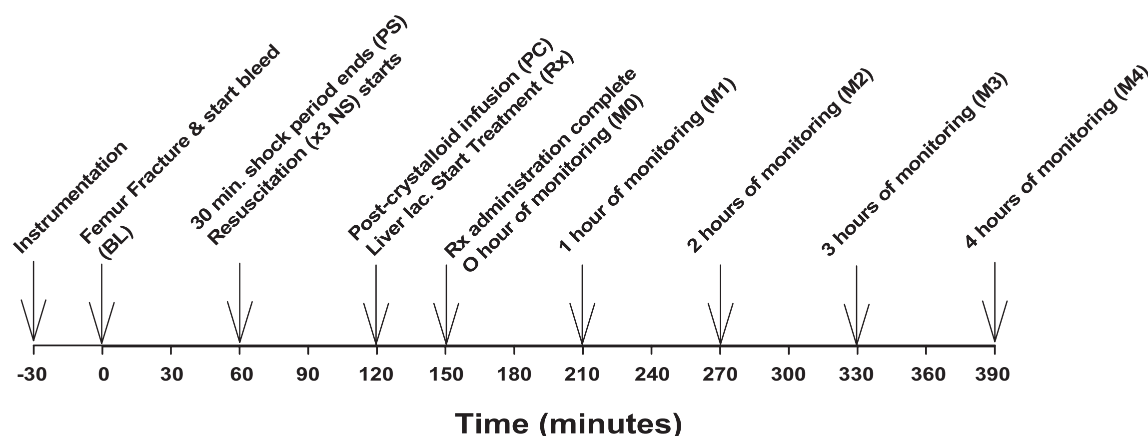

Fig. 1. *Timeline of the multiple injuries or hemorrhagic shock model. NS, normal saline, Rx, treatment.*

output. At this point, anesthesia was adjusted to allow animals to reach a mean arterial pressure (MAP) of 60 mm Hg or more. This marked the end of instrumentation phase and at this point, samples for the time-point BL were drawn.

Monitoring

Noninvasive monitoring included pulse oximetry and continuous electrocardiography. An esophageal thermometer was inserted to measure core temperature. Invasive hemodynamic monitoring (central venous and arterial pressures) was continuously performed (Eagle 4000 Patient Monitor, GE Marquette, Piscataway, NJ), and blood pressure readings were recorded every 5 minutes. End-tidal CO_2 was also measured throughout the experiment (V9004, Surgivet, Waukesha, WI) along with respiratory rate.

Blood Sampling and Analysis

Arterial blood samples were collected at BL to measure PT, PTT, INR, fibrinogen, thromboelastography (TEG), complete blood count, and arterial blood gas. These measurements were performed at 8 time points during the experiment (Fig. 1): BL, postshock, postcrystalloid infusion (PC), end of administration of treatment (M0), after 1 hour of monitoring (M1), after 2 hours of monitoring (M2), after 3 hours of monitoring (M3), and after 4 hours of monitoring (M4). A full description of these time points is presented in later sections. PT, PTT, INR, and fibrinogen were all measured using the STart 4 machine (Diagnostica Stago, Parsippany, NJ). TEG was performed using the TEG 5000 Thromboelastograph Hemostasis Analyzer (Hemoscope, Niles, IL) using citrated kaolin samples and with temperatures adjusted to match the core temperature of the swine at the time of sample collection. Arterial blood gas analysis was performed using a Stat Profile 2 Blood Gas and Electrolyte Analyzer (Nova Biomedical, Waltham, MA).

Femur Fracture and Soft Tissue Injury

The left mid-shaft femur was localized using a 14-gauge needle, followed by a cruciate incision at the site of localization, after which a captive bolt gun (Model RS22, Ramset—Powder Fastening System, Glendale Heights, IL) was applied to the femur and fired at the site to induce both a soft tissue injury and femur fracture. The mid-shaft of femur was palpated for confirmation of a fracture. Figure 2, A is a 3D computed tomography reconstruction of the type of femur fracture created by this method. Bleeding from the site was measured and subtracted from total hemorrhage volume.

Hemorrhage and Resuscitation Protocol

Total blood volume was estimated,[20] and 60% of it was withdrawn using a Masterflex pump Model L/S Computerized Drive with a MF EasyLoad II Pumphead, Model 77201–60 (Cole Palmer, Vernon Hills, IL). Blood was withdrawn at a rate of 100 mL/min, and was captured in a Terumo 3-bag blood collection set (CPDA and AS-5) (Terumo Products, Somerset, NJ). Isoflurane was decreased with falling blood pressure and turned off at a MAP of 30 mm Hg. Hemorrhage was also held briefly for MAP $<$25 mm Hg and 0.9% normal saline (NS) (Baxter Healthcare Corporation, Deerfield, IL) was administered intravenously at a rate of 165 mL/min using Masterflex pump into the internal jugular vein. Once MAP reached 30 mm Hg, infusion was stopped and hemorrhage was reinitiated. Thus, the swine was kept within a MAP of 25 mm Hg to 30 mm Hg until 60% of blood volume was withdrawn. After hemorrhage, the animal was left in shock for 30 minutes (representing time in the field before medical attention). The end of this period represented the postshock time point.

After 30 minutes of shock, the lost blood was replaced with three times the volume of NS (room temperature) at a rate of 165 mL/min using the Masterflex pump through the internal jugular vein catheter. The amount of saline given during the hemorrhage phase was taken into account while calculating the volume of replacement saline. After resuscitation, animals were allowed to equilibrate for 15 minutes.

Mild Hypothermia

By the end of the 15-minute equilibration period after crystalloid infusion, most of the animals had spontaneously become hypothermic. However, if target temperature of

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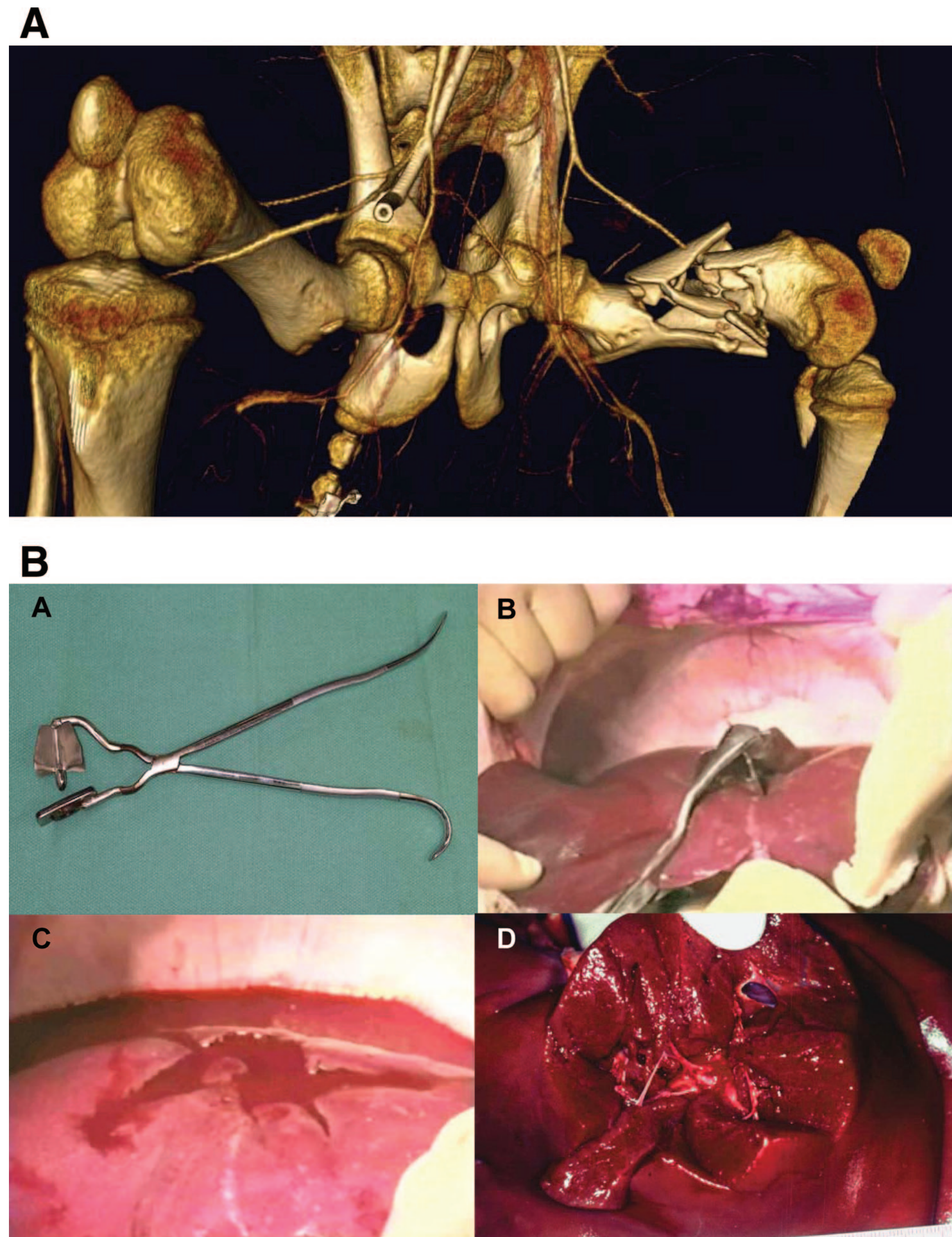

Fig. 2. *Injuries.* Panel A *shows a 3D reconstruction computerized tomography image of the femur fracture (provided by Dr. Jill Sondeen).* Panel B *shows the grade V liver injury (*A*, device;* B*, Injury being created;* C*, actively bleeding liver laceration;* D*, postmortem view of the injury showing lacerated vascular structures).*

33.0°C ± 0.3°C was not achieved spontaneously, intraperitoneal lavage with chilled crystalloid was performed to reach that temperature. At this stage, all of the animals were equally hypothermic, and had developed lactic acidosis and coagulopathy. This was designated as the PC infusion time point.

Liver Laceration

After 15 minutes of observation, preweighed laparotomy sponges were placed in both paracolic gutters and in the pelvis for blood collection. A grade V liver injury (simulating delayed rupture of a contained injury) was created using a specialized clamp (Fig. 2, B).[21] After the laceration, the liver was left to freely bleed for 30 seconds followed by packing with six dry laparotomy pads (4 superior and 2 inferior). Abdomen was then closed using a running suture. This marked the end of the last phase of the trauma or hemorrhage protocol and animals were randomized into four groups (n = 6/group): No treatment (control), FWB, FFP, FDP.

Treatment Protocol

Immediately after liver packing, transfusion of different blood products was started. The volume of transfusion

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matched the volume of withdrawn blood (60% estimated blood volume) and it was infused at a rate of 50 mL/min using the Masterflex pump. For those products that had been citrated, 50 mmol/L of $CaCl_2$ was also infused over the same time period through the external jugular venous line. All the blood products were warmed in a 38°C water bath before administration.

Monitoring and Tissue Harvesting

Animals were monitored for 4 hours and blood samples were collected every hour. All the animals that survived for 15 minutes after completion of the treatment were included in the study. All control animals that survived for 15 minutes after the liver injury were included. At the end of the monitoring period, or at the time of death, laparotomy sponges were weighed for contained blood. Tissue samples from lung, liver, kidney, and small bowel (distal ileum) were harvested for analysis at a later stage, and placed in liquid nitrogen, 10% paraformaldehyde and RNAlater (Ambion, Inc., Austin, TX). Frozen tissues were then stored at −70°C and RNA later was placed in 4°C for 24 hours followed by permanent storage at −20°C. After tissue harvest, the animal was killed using Euthasol (sodium pentobarbital [100 mg/kg]).

Blood Products Preparation

Transfused plasma was obtained from autologous and donor blood. After withdrawal, blood was spun for 15 minutes at 5,000 rpm with no brake (Model J-68 Centrifuge, Beckman Coulter, Fullerton, CA) to separate the plasma from other components. Platelet poor plasma was then extracted (Fenwal Plasma Extractor, Baxter, Deerfield, IL) and collected in the Terumo blood system satellite bag. It was stored in a −70°C freezer if not scheduled for immediate use. This frozen plasma was either thawed for transfusion, or lyophilized to develop the FDP. Blood products were transfused through a transfusion kit with a filter (V2500, Y-Type Blood Set—170 μmol/L Blood Filter, Braun, Bethlehem, PA).

Table 1 In Vitro Comparison of Fresh Frozen Plasma and Freeze Dried Plasma

Parameter	FFP (n = 10)	FDP (n = 10)	*p*
PT (s)	13.4 ± 0.6	13.5 ± 0.3	NS
INR	1.15 ± 0.17	1.15 ± 0.11	NS
PTT (s)	23.1 ± 2.1	26.5 ± 2.7	NS
Fibrinogen (mg/dL)	122.5 ± 18.0	120.6 ± 15.7	NS
Factor II activity level (%)	22.5 ± 2.1	25.4 ± 4.5	NS
Factor VII activity level (%)	16.2 ± 1.7	16.4 ± 4.5	NS
Factor IX activity level (%)	250.6 ± 24.0	201.8 ± 51.5	NS

Data shown as means ± standard error of the mean.
NS, no significant difference ($p > 0.05$).

Statistical Analysis

All data are presented as group means ± SEM. The SPSS statistical software program (SPSS/Windows, SPSS Inc) was used. Groups were compared by one-way analysis of variance with Dunnett's test for multiple comparisons on all continuous variables, whereas Fisher's exact test was used to compare nominal data. Statistical significance was defined as $p < 0.05$.

RESULTS

In Vitro Analysis

In vitro comparison of FFP and FDP using standard coagulation tests (PT, INR, PTT, and Fibrinogen) did not reveal any statistically significant differences. The levels of activity of selected clotting factors were also similar (Table 1).

Survival

The BL characteristics for all the groups are shown in Table 2. There were no differences between the groups at BL. This was a highly lethal model, as only one animal survived in the control group (16% survival). All the other control animals (given crystalloids but no blood products) died before the M1 time point, with an average time to death of 55 minutes ± 3 minutes after liver injury. All the animals treated with blood products (FWB, FFP, and FDP) survived the entire monitoring period.

Hemodynamic Data

MAP at the end of hemorrhage (PH) decreased to less than 30 mm Hg ($p < 0.001$ compared with BL) and remained less than 40 mm Hg at the end of the shock period ($p < 0.01$ compared with BL). Transfusion with FWB, FFP, and FDP increased the MAP to near-BL levels in these groups for 2 hours, but by M4 their MAP had declined to less than 50

Table 2 Baseline Comparison of Different Treatment Groups

Variable	Control (n = 6)	Fresh Whole Blood (n = 6)	Fresh Frozen Plasma (n = 6)	Freeze Dried Plasma (n = 6)	*p*
Weight (kg)	42.8 ± 2.2	43.7 ± 1.1	42.8 ± 1.6	42 ± 2.4	NS
Blood loss (mL/kg)	42.1 ± 0.4	41.8 ± 0.4	41.6 ± 0.5	42.6 ± 0.5	NS
IVF (mL/kg)	126.3 ± 1.4	124.4 ± 0.6	123.7 ± 0.7	127.8 ± 1.4	NS
Hct (%)	27.8 ± 1.6	26.4 ± 1.9	30.8 ± 1.1	27.0 ± 1.1	NS
Platelets ($\times 0^3$/L)	263.3 ± 22.9	328.5 ± 55.2	246.3 ± 18.3	337.0 ± 64.1	NS
Lactate (mmol/L)	0.9 ± 0.2	2.3 ± 1.0	1.1 ± 0.3	1.2 ± 0.2	NS
Base excess (mmol/L)	2.0 ± 1.3	2.0 ± 1.0	4.3 ± 0.8	4.5 ± 1.1	NS

Data shown as means ± standard error of the mean.
NS, no significant difference ($p > 0.05$); IVF, intravenous fluids; Hct, hematocrit.

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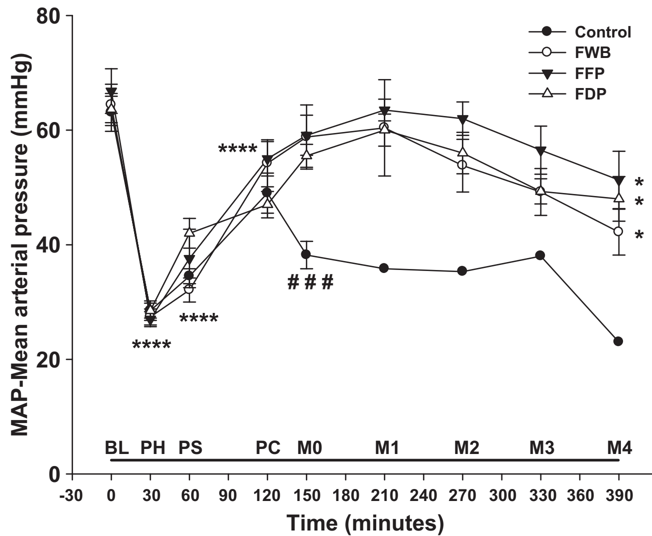

Fig. 3. *Mean arterial pressures (MAP). Data are presented as group means ± SEM.* *p < 0.05 compared with baseline. #p < 0.05 compared with control. FWB, fresh whole blood; FFP, fresh frozen plasma; FDP, freeze-dried plasma; BL, baseline; PH, posthemorrhage; PS, postshock; PC, postcrystalloid; M0, end of treatment administration; M1, 1 hour after treatment; M2, 2 hours after treatment; M3, 3 hours after treatment; M4, 4 hours after treatment.*

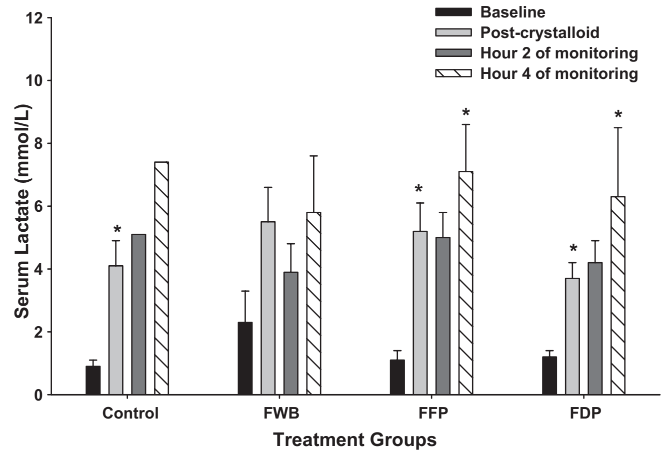

Fig. 4. *Serum lactate at four time points. Data are presented as group means ± SEM.* *p < 0.05 compared with baseline. There were no significant intergroup differences. FWB, fresh whole blood; FFP, fresh frozen plasma; FDP, freeze-dried plasma.*

($p < 0.05$ compared with BL). The MAP in the control group was lower than the other three groups at M0 (38 mm Hg vs. >55 mm Hg, $p < 0.05$) and the only animal that survived without treatment continued to follow a declining trend from M1 onwards, until M4 when it was killed with a MAP of 23 mm Hg (Fig. 3). In this model, hemorrhage and infusion of crystalloids resulted in the development of significant lactic acidosis, dilutional anemia, and thrombocytopenia (Table 3). As expected, treatment with FWB, but not FFP and FDP corrected the anemia and thrombocytopenia (Table 3). Lactic acidosis persisted despite treatment, and it actually worsened in the FDP and FFP treated animals (Fig. 4), most likely as a result of decreased tissue oxygen delivery because of the anemia.

Comparison of PT/INR, PTT, Fibrinogen, and TEG

After trauma, hemorrhage and crystalloid infusion, all of the animals developed significant coagulopathy (PT/INR increase of ~1.5 fold). Treatment with FWB, FFP, and FDP corrected the PT/INR rapidly (immediately after treatment, M0), and the effect lasted for the entire period of monitoring (M0 through M4). The control group had higher PT/INR values at M0 compared with the resuscitation groups (16.5 vs.

Table 3 The Effect of Shock Protocol and Resuscitation Strategies on Selected Parameters

Parameter	Group	Baseline	Postcrystalloid	4 h of Monitoring (M4)
Hct (%)	Ctrl	27.85 ± 1.6	11.85 ± 0.7*	15
	FWB	26.45 ± 1.9	15.75 ± 0.9*	28.25 ± 2.1
	FFP	30.85 ± 1.1	16.15 ± 1.3*	15.25 ± 0.9*
	FDP	27.05 ± 1.1	15.35 ± 1.2*	15.65 ± 2.4*
Lactate (mmol/L)	Ctrl	0.95 ± 0.2	4.15 ± 0.8*	7.4†
	FWB	2.35 ± 1.0	5.55 ± 1.1	5.85 ± 1.8
	FFP	1.15 ± 0.3	5.25 ± 0.9*	7.15 ± 1.5*
	FDP	1.25 ± 0.2	3.75 ± 0.5*	6.35 ± 2.2*
Base Excess (mmol/L)	Ctrl	2.05 ± 1.3	−5.05 ± 1.7*	−7.1†
	FWB	2.05 ± 1.0	−8.65 ± 1.5*	−7.35 ± 2.4*
	FFP	4.35 ± 0.8	−7.65 ± 1.5*	−2.85 ± 2.9*
	FDP	4.55 ± 1.1	−5.95 ± 1.2*	−3.85 ± 1.5*
Platelets ($\times 10^3$/fl)	Ctrl	263.35 ± 22.9	157.15 ± 3.71	141
	FWB	328.55 ± 55.28	180.25 ± 33.8*	280.05 ± 32.0*
	FFP	246.35 ± 18.26	109.65 ± 17.8	176.05 ± 33.6
	FDP	3375 ± 64.12	175.35 ± 34.5*	165.35 ± 40.3*

Data shown as means ± standard error of the mean.
* $p < 0.05$ compared to baseline. There were no Intergroup differences. Ctrl, no treatment.
† Value is from M3 time point because of an error in analyzing the M4 sample.

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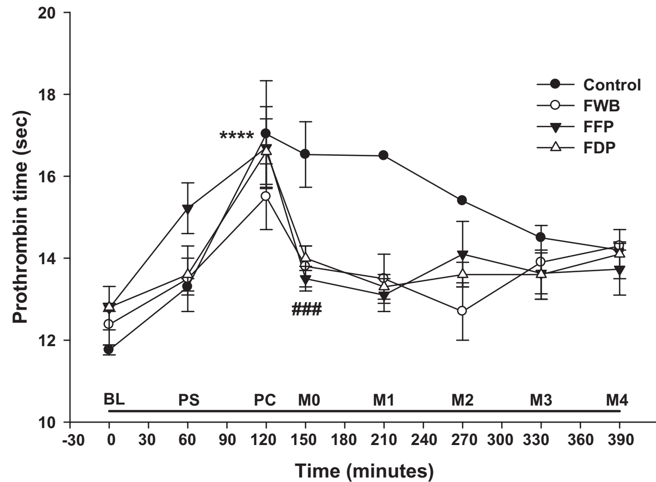

Fig. 5. *Prothrombin time. Data are presented as group means ± SEM. *p < 0.05 compared with baseline. #p < 0.05 compared with control. FWB, fresh whole blood; FFP, fresh frozen plasma; FDP, freeze-dried plasma; BL, baseline; PH, posthemorrhage; PS, post-shock; PC, postcrystalloid; M0, end of treatment administration; M1, 1 hour after treatment; M2, 2 hours after treatment; M3, 3 hours after treatment; M4, 4 hours after treatment.*

<14, $p < 0.05$). The only control animal that survived showed normalization of PT and INR; however the reversal was much slower (Figs. 5 and 6). There was no significant difference in aPTT values for any group at any point in this model (data not shown). The fibrinogen concentrations in the plasma after crystalloid resuscitation dropped below detectable levels. This was most likely due to the dilution produced

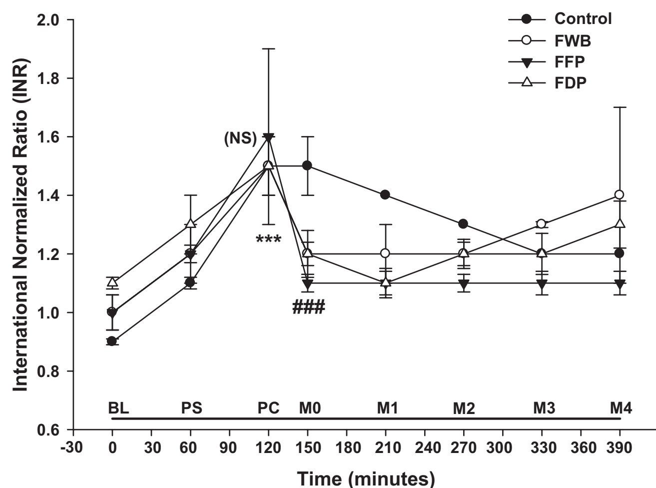

Fig. 6. *International Normalized Ratio (INR). Data are presented as group means ± SEM. *p < 0.05 compared with baseline. #p < 0.05 compared with control. NS, no significant difference from baseline; FWB, fresh whole blood; FFP, fresh frozen plasma; FDP, freeze-dried plasma; BL, baseline; PH, posthemorrhage; PS, post-shock; PC, postcrystalloid; M0, end of treatment administration; M1, 1 hour after treatment; M2, 2 hours after treatment; M3, 3 hours after treatment; M4, 4 hours after treatment.*

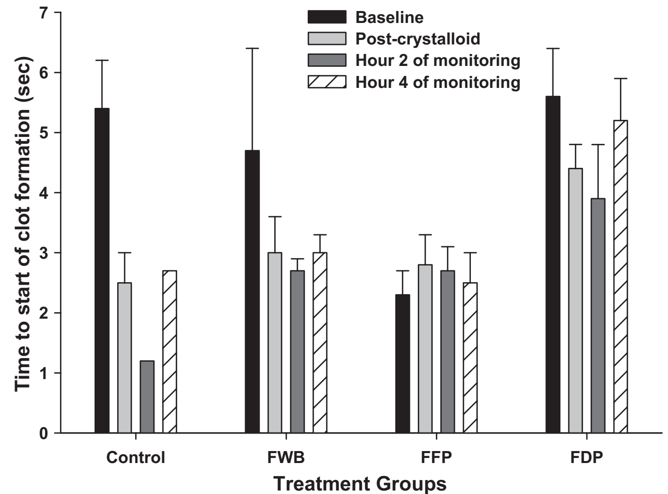

Fig. 7. *Time to clot formation (Thromboealstography— 'r' time) at four time points. Data are presented as group means ± SEM. None of the differences reached statistical significance. FWB, fresh whole blood; FFP, fresh frozen plasma; FDP, freeze-dried plasma.*

by the intravenous fluid administration, and hence, fibrinogen levels were not included in our final analysis.

All groups had similar TEG values at BL except for FFP, which had a shorter time to start of clot formation ("r" time), although this was not statistically significant. After completion of our study protocol and administration of treatment, there was a consistent trend toward a decrease in the "r" time for all the groups, suggesting faster onset of clot formation, except FFP, which showed a slight increase. None of these values reached statistical significance (Fig. 7). There was a decrease in the rate of clot formation (α) after crystalloid resuscitation (PC) in all the groups. This was corrected in all the four groups (data not shown). There was a decrease in the maximum strength of the clot (MA) after crystalloid infusion (PC), which was statistically significant in all groups but FFP ($p = 0.08$). The MA remained low in the control group, but all the treatment groups showed return of clot strength back to normal by the end of the observation period (Fig. 8).

DISCUSSION

Trauma-associated coagulopathy is multifactorial in pathogenesis. Hypothermia causes platelet dysfunction[2] and decreases the functional activity of clotting factors.[3] A drop in temperature (<33°C) decreases the functional activity of clotting factors below 50%.[4–7] The enzyme complexes involved in clotting cascade are pH sensitive, and are profoundly influenced by the degree of metabolic acidosis.[6] In a swine model, acidosis (pH = 7.1) alone has been shown to increase the splenic bleeding time by 41%, and by 72% when combined with hypothermia (T = 32°C).[7] Furthermore, dilution of clotting factors (from crystalloid and packed red cell resuscitation) can lead to coagulopathy. A large retrospective review of over 17,000 trauma patients revealed that nearly half of the patients who received more than 2 L of prehospital

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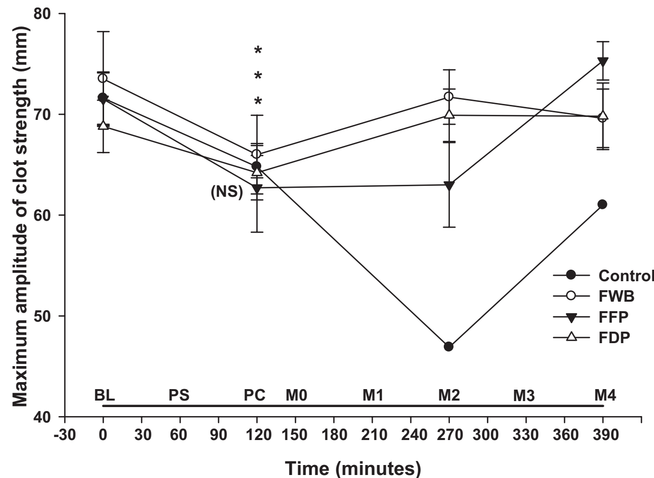

Fig. 8. *Maximum Amplitude (Thromboealstography-MA value) at four time points. Data are presented as group means ± SEM.* *$p < 0.05$ *compared with baseline. NS, no significant difference from baseline; FWB, fresh whole blood; FFP, fresh frozen plasma; FDP, freeze-dried plasma; BL, baseline; PH, posthemorrhage; PS, post-shock; PC, postcrystalloid; M0, end of treatment administration; M1, 1 hour after treatment; M2, 2 hours after treatment; M3, 3 hours after treatment; M4, 4 hours after treatment.*

fluids were coagulopathic on arrival.[8] Besides the volume, choice of resuscitation fluid also determines the degree of ensuing coagulopathy. In a swine model of hemorrhagic shock, Kiraly et al.[9] have reported that resuscitation with NS resulted in a hypocoagulable state and increased blood loss as compared with resuscitation with lactated Ringers (LR) solution. Similarly, large volume infusion of hetastarch solution along with hypothermia produces coagulopathy.[22] Brohi et al. have recently implicated the Protein C pathway in the development of hypoperfusion-mediated coagulopathy. Using base deficit as a marker of tissue perfusion, they concluded that early coagulopathy develops only in the presence of tissue hypoperfusion (base deficit >6 mEq/L). They also observed increased D-dimer and tissue plasminogen activator levels in the trauma patients, suggesting that anticoagulation or hyperfibrinolysis also plays a part in trauma-associated coagulopathy.[10]

Keeping in mind the diverse pathophysiology of trauma-associated coagulopathy, we designed a complex multiple injuries model that would include most, if not all of the risk factors: long bone fracture, soft tissue injuries, large volume blood loss, period of tissue hypoperfusion, hypothermia, acidosis, hemodilution, open body cavity, and delayed hemorrhage from a solid organ injury. Although all of the animals developed hypothermia because of shock, peritoneal irrigation with ice-cold saline was required in 25% of the animals to bring the core temperature down to 33°C. This should be kept in mind while analyzing our findings, as induced and spontaneous hypothermia are physiologically different. The model was highly lethal, with 85% mortality in the control group that received crystalloids (3 times the volume of shed blood), but no blood products. During the model development phase, it was noticed that allowing the MAP to fall below 25 mm Hg resulted in irreversible shock and 100% mortality despite treatment, whereas maintaining it between 25 mm Hg and 30 mm Hg was found to consistently result in substantial tissue hypoperfusion while still allowing treatment to alter the outcome. All groups developed significant metabolic acidosis after the hemorrhage or trauma protocol (Table 3 and Fig. 4). This acidosis persisted during the 4 hours of observation, which may be due to the severity of the insult or the relatively short observation period (clinically it can take up to 24 hours postresuscitation to clear significant lactic acidosis after multiple injuries). Also, the plasma treated groups (FFP and FDP) developed anemia (Table 3), which clearly has an influence on the tissue oxygen delivery and the rate of lactic acid clearance. We reasoned that FDP could be used for the treatment of coagulopathy in the prehospital arena, and the anemia could be corrected with red blood cell (RBC) transfusions after reaching a higher level of care. Comparison of different treatment strategies in this model revealed that infusion of the three different plasma rich products (FWB, FFP, and FDP) rapidly corrected the PT/INR values. Immediately after the treatment agent had been administered (M0), most of the animals were close to their preinjury PT/INR and remained so for the next 4 hours. None of these animals died during the observation period. However, in the control group only one animal lived beyond 1 hour (85% mortality), and gradually autocorrected the coagulation profile.

In addition to the typical parameters, another tool used in our study to measure the coagulation profile was TEG. This technology measures the visco-elastic properties of whole blood and is used frequently in cardiac and transplant operations. TEG provides functional information about the interaction between platelets and clotting factors, ranging from platelet aggregation to eventual clot lysis. A TEG tracing can be divided into several components, which have been explained in great detail in several studies.[23] Briefly, the most commonly used variables in the tracing are "r," "α," and "MA." The "r" denotes reaction time, which is defined as the time to onset of clot formation. A prolonged "r" represents clotting factor deficiency. The "α" angle represents the rate of development of clot strength after initiation of clot formation. It is dependant on rate of fibrin build-up and cross-linking. The "MA" measures maximum clot strength. It reflects primarily platelet function, and to a lesser degree, fibrinogen concentration. There is a growing interest in the use of TEG in trauma, although differences exist in the definition of a "hypocoagulable" TEG tracing. This disagreement stems from the fact that different segments of a TEG tracing represent different components of hemostasis, with some overlap. Moreover, they respond differently to various factors, which often leads to a mixed picture. For example, acidosis has minimum effect on MA, while there is a strong correlation between pH and α.[24] Hypothermia and hemodilution decrease both MA and α.[22] Resuscitation with saline is asso-

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ciated with a higher "r" and smaller MA (hypocoagulability), but a higher α (hypercoagulability).[9] In addition, complexity originates from the effect of the TEG cuvette materials on hemostatic processes, such as platelet activation and turnover of the intrinsic coagulation cascade. Because of these issues, the use of TEG in trauma patients is not completely straightforward. A prospective study of coagulation changes in critically ill trauma patients over 4 days showed a shortened "r" value (faster onset of clot formation) on day 1 compared with day 4 (62% vs. 26%), leading the authors to conclude that trauma patients are hypercoagulable acutely after trauma.[25] However, in that study MA and α remained within normal limits and there was a poor correlation between TEG results and the standard coagulation tests (PT, INR, aPTT). Another prospective study of 69 blunt trauma patients defined hypocoagulability or hypercoagulability as abnormality (increase or decrease) of two of four selected TEG parameters.[26] They found 45 patients (65%) to be hypercoagulable by that definition, and only the Injury Severity Score and TEG were predictive of transfusion requirement within the first 24 hours. The TEG data from our study showed some interesting results. Most of the animals demonstrated a shortened reaction time ("r" time) by the time of completion of our shock or trauma protocol, which is consistent with the clinical literature.[25,26] The only exception was the FFP group that had a lower "r" time at BL and, therefore, could not gain much in faster clot formation after treatment with FFP. In addition, the overall trend for "r" time from BL to pretreatment was opposite to the PT/INR data, which showed development of a significant coagulopathy. Although Schreiber et al.[25] have shown significant correlation between "r" time and PTT, this was not seen in our model (data not shown). This apparent contrast between PT/INR and TEG "r" time is an interesting finding, since PT/INR measure the start of clot formation (via the extrinsic pathway), the same process calculated by "r." Whether this represents true hypercoagulability that could potentially lead to thromboembolic events, is unknown. There are several mechanisms whereby trauma could lead to a hypercoagulable state.[27,28] Tissue damage and activated platelets release thromboplastic substances such as thromboxanes, epinephrine, serotonin, and adenosine, all of which cause vasoconstriction and promote platelet-fibrin interaction. The other two TEG parameters measured by our group displayed attenuated clotting with the trauma or hemorrhage protocol: there was a significant decrease in the strength of the clot (MA), and the rate of clot formation (α) followed a similar trend although it did not reach statistical significance. Overall, there was a statistically significant drop in MA, except in the FFP group, at the postcrystalloid infusion (PC) time point. An interesting finding was that the animals treated with FFP and FDP were able to form a clot at the end of the experiment that was as strong (if not stronger) as the BL (Fig. 8). Classically, MA is attributed more to platelet function than to fibrinogen,[23] but our data suggest that this may not be entirely true as the platelet count in the plasma groups was very low. One possible explanation is that the FFP and FDP might supply enough additional fibrinogen so that, with remaining endogenous platelets as focus points for fibrin polymer organization, stiffer clots with more native visco-elastic properties can be formed.

In this experiment, we included the FWB treatment group as a control because of its increasing use in the combat environment.[16,29] A review of transfusion practices in Operation Iraqi Freedom has revealed that 13% of patients received FWB.[16] Spinella et al.[30] studied 87 patients with 545 units of FWB transfusion at one combat hospital and reported significant improvement in both hemoglobin concentration and coagulation parameters with the use of FWB. Supporters of FWB claim that it acts as a ready source of RBCs, platelets, and clotting factors. The total concentration of these components when given as FWB is significantly higher than when each is given as a separate unit.[16] Also, FWB does not suffer from the problems of RBC storage, such as metabolic depletion and membrane loss. Safety of this practice is clearly an issue, but a retrospective review of nearly 3,000 units of FWB donated in Afghanistan and Iraq revealed only three positive tests for hepatitis C antibody and one for human T lymphocyte virus.[31] FWB transfusion requires availability of volunteer donors who are prescreened and typed.[17] Such constraints associated with FWB limit its use primarily to combat situations, and it is unlikely to gain wide acceptance in the civilian setting. The other two strategies used to treat coagulopathy in this experiment were plasma based, which is widely accepted in clinical practice. A number of recent studies have proposed that trauma-associated coagulopathy should be managed with large volume infusion of FFP (higher ratios of FFP to packed red blood cell).[14,15,32,33]

Although the optimal dose may be debated, based on computer simulation of dilutional coagulopathy[15] and clinical literature,[32,33] the role of FFP in the treatment of coagulopathy is beyond doubt. But just like FWB, it too has logistical shortcomings. FFP needs to be stored at $-18°C$ and its maximum storage life is 1 year.[17] It must be kept refrigerated during transportation, and thawed just before use (~30 minute preparation time). These characteristics make it less than ideal for the combat environment, where appropriate storage facilities are not available at all echelons of care, particularly close to the battlefield. These logistical problems could be solved by the development of a product with a long shelf life that is stable at higher temperatures, is easily rehydrated, has ABO universality, and displays a good safety profile. We propose that lyophilized FDP may be such a product.

Lyophilized plasma has been used for bleeding control since World War II with the first clinical reports appearing in the literature in the 1950s. Oktavec and Smetana assessed the reliability and longevity of lyophilized plasma, which was used as a control for calculating the PT. Comparison of lyophilized plasma with fresh plasma from healthy individuals has shown a high degree of correlation,[34] and it has been used to treat bleeding episodes in hemophiliac patients.[35,36] It

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has also been demonstrated that after a year of storage at −25°C, lyophilized plasma has the same clotting factor stability as its fresh counterpart.[37] Plasma proteins are heat labile, hence the need to keep FFP at such low temperatures. In a very important study, Ramsey et al.[38] studied the effects of heat treatment, as part of viral inactivation methods, on lyophilized plasma. Samples of lyophilized plasma were heated in a water bath at 60°C for 1 hour, 4 hours, 8 hours, 24 hours, and 72 hours before reconstitution. Heating fresh plasma or lyophilized plasma after reconstitution significantly prolonged PT for these samples. However, nonreconstituted lyophilized plasma showed no change in PT after as long as 24 hours of heating, with only a mild increase after 48 hours and 72 hours. Factor V activity decreased after heating (60% after 8 hours and 48% after 24 hours), but the activities of Factor VII, VIII, and IX exhibited minimum deviations from their unheated levels. The preservation process is remarkably effective, and storage of lyophilized plasma for as long as 30 years fails to alter its components, including hormones, enzymes, and other proteins.[39] Despite these attractive features, use of freeze-dried lyophilized plasma fell out of favor during the 1960s and 1970s because of concerns about the inability of the lyophilization process to inactivate viruses present in pooled plasma.[40] Viral inactivation methods have evolved since then and the risk of Human Immunodeficiency Virus or Hepatitis C Virus transmission in blood component therapy has dropped to one in two million units.[41] The modified solvent or detergent treatment for human plasma inactivates $>10^6$ chimpanzee-infectious doses (CID_{50}) of Hepatitis B Virus and $>10^5$ CID_{50} of Hepatitis C Virus.[42] The same techniques are now used for viral inactivation during the production of the FDP.

Our in vitro testing essentially reproduced the results that Oktavec and Smetana[34] reported more than 50 years ago. There was no difference in the coagulation parameters (PT, PTT, INR, Fibrinogen) between the fresh and freeze-dried plasmas. Measurement of clotting factors selected to represent the extrinsic, intrinsic, and common arm of the coagulation cascade[1] revealed only a mild (and insignificant) decrease in the activity of coagulation factors II, VII, and IX in the FDP samples. It should be pointed out that as this was a feasibility study, we did not measure all of the proteins involved in the coagulation cascades. Similarly, we also did not measure the levels of proteins involved in inhibition of fibrinolysis (e.g., plasminogen activator inhibitor), as well as different antithrombotic proteins (e.g., proteins C and S). These are being measured in the follow-up experiment that is currently ongoing.

In conclusion, our data supports the fact that plasma can be lyophilized into a freeze-dried formulation without compromising the level and function of clotting factors. This product offers logistical advantages, and it is as effective as FFP in reversing coagulopathy in a poly trauma and shock model.

ACKNOWLEDGMENTS

We thank Martin Schreiber, MD (Oregon Health and Science University, Portland, OR) and John Holcomb, MD (US Army Institute of Surgical Research, San Antonio, TX) for their invaluable help in developing the animal model (funded by the US Army Medical Research and Materiel Command). We also thank Jill Sondeen, PhD (US Army Institute of Surgical Research, San Antonio, TX) for her guidance in setting up the coagulation tests. Irina Bakaltcheva, PhD (Walter Reed Army Institute of Research, Silver Spring, MD) developed the process for making the lyophilized freeze-dried plasma, and Entegrion Inc. (Research Triangle Park, NC) provided the infrastructure for its actual production. Yongqing Li, MD, PhD and Baoling Liu, MD helped with the measurement of coagulation profiles. Mentorship provided by Dr. Peter Rhee is also acknowledged with deep appreciation. The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense at large.

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