PAPER

Lyophilized Plasma for Resuscitation in a Swine Model of Severe Injury

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Hypothesis: Lyophilized plasma (LP) is as safe and effective as fresh frozen plasma (FFP) for resuscitation after severe trauma.

Design: Multicenter animal study.

Setting: Animal laboratories, 2 level I trauma centers.

Participants: Thirty-two Yorkshire crossbred swine.

Interventions: Lyophilized plasma was analyzed for factor levels and clotting activity before lyophilization and after reconstitution. Swine were subjected to complex multiple trauma including extremity fracture, hemorrhage, severe liver injury, acidosis, and hypothermia. They were then resuscitated with FFP, LP, FFP and packed red blood cells (PRBCs) in a ratio of 1:1, or 1:1 LP and PRBCs.

Main Outcome Measures: Residual clotting activity of LP after reconstitution, swine mortality, hemodynamic measures, total blood loss, coagulation profiles, and inflammatory measures.

Results: Lyophilization decreased clotting factor activity by an average of 14%. Survival and heart rate were similar between all groups. Swine resuscitated with LP had equivalent or higher mean arterial pressures. Swine treated with LP had similar coagulation profiles, plasma lactate levels, and postinjury blood loss compared with those treated with FFP. Swine treated with 1:1 FFP-PRBCs were similar to those treated with 1:1 LP-PRBCs. Resuscitation with LP resulted in a reduction in postresuscitation interleukin 6 expression compared with resuscitation with FFP.

Conclusions: The process of lyophilization and reconstitution of plasma reduces coagulation factor activity by 14%, without acute differences in blood loss. Lyophilized plasma can be used for resuscitation in a severe multiple trauma and hemorrhagic shock swine model with efficacy equal to that of FFP and with decreased interleukin 6 production.

Arch Surg. 2009;144(9):829-834

TRAUMATIC INJURY IS THE LEADing cause of death in the United States for persons between the ages of 1 and 34 years.[1] As a mainstay of trauma care, early and effective hemostatic resuscitation holds great potential for restoring lost blood volume, restoring tissue perfusion, correcting coagulopathy, and saving lives. Optimal methods of achieving these goals are a subject of ongoing debate.

Current practices call for an initial crystalloid infusion followed by blood products when needed. In severely injured patients, hemorrhage often results in the need to transfuse blood. Recent studies in severely injured patients have shown that using ratios of fresh frozen plasma (FFP) to packed red blood cells (PRBCs) approaching 1:1 improves mortality[2-10] and coagulopathy in massively transfused patients.[11]

The cumbersome storage requirements of FFP make it difficult to achieve these high ratios in many centers. Specifically, FFP must be kept frozen until just before use and thawed slowly by using a warm-water bath or specially designed microwave oven. This prevents the use of FFP for resuscitation in far-forward combat situations and in the prehospital setting, and limits its early use in many civilian centers. Lyophilized plasma (LP) can be stored at room temperature, easily transported, and quickly reconstituted. An initial study of LP by Shuja et al[12] showed encouraging results with the use of a freeze-dried form of plasma for resuscitation. The present study takes those initial investigations further by evaluating the effects of the lyophilization process on plasma clotting factor levels, by adding the antioxidant ascorbic acid (vitamin C) to the reconstitution solution, and by comparing the efficacy of LP with that of FFP and that of plasma and PRBCs in a 1:1 ratio.

We hypothesized that the lyophilization process followed by reconstitution would result in a minimal decrease in clotting factor activity. We also hypothesized that lyophilized plasma would have effects on physiology and coagulopathy correction similar to those of FFP in a severe multisystem hemorrhagic shock swine model. Finally, because of the addition of ascorbic acid in the reconstitution fluid, we hypothesized that resuscitation with LP would suppress dysfunctional inflammation.

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Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 SEP 2009	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Lyophilized Plasma for Resuscitation in a Swine Model of Severe Injury.	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Spoerke N., Zink K., Cho S. D., Differding J., Muller P., Karahan A., Sondeen J., Holcomb J. B., Schreiber M.,	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Institute of Surgical Research, JBSA Fort Sam Houston, TX 78234	
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**	**6**	

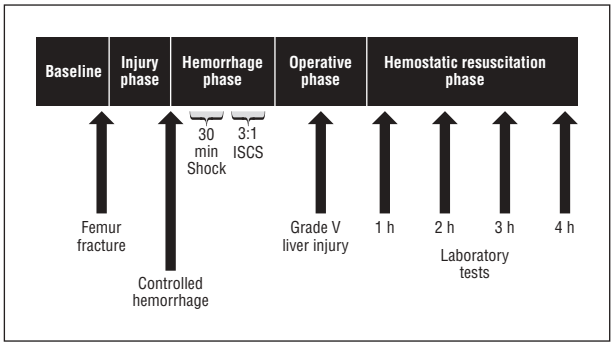

Figure 1. Swine model of severe injury and hemorrhagic shock. 3:1 ISCS indicates isotonic sodium chloride solution at a volume 3 times the controlled hemorrhage volume.

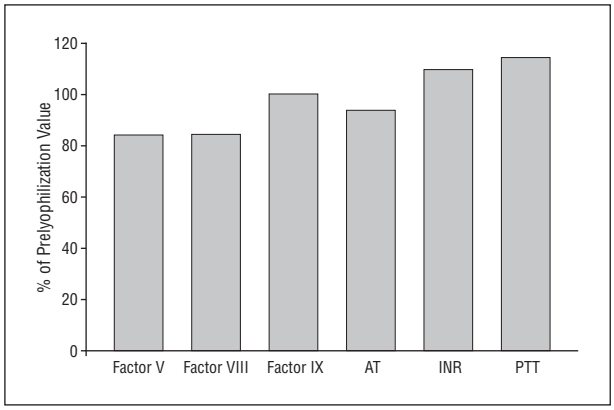

Figure 2. Clotting factor activity and coagulation assays comparing the postreconstitution value with the prelyophilization value. Values are expressed as means. AT indicates antithrombin III; INR, international normalized ratio; and PTT, partial thromboplastin time.

METHODS

PREPARATION OF PLASMA FOR LYOPHILIZATION

All experimental procedures were done in accordance with the guidelines of the Institutional Animal Care and Use Committee at Oregon Health and Science University and the US Army Institute of Surgical Research. Blood products used in the study were obtained from juvenile Yorkshire crossbred swine. With the use of sterile precautions, a cervical cutdown was performed and the external jugular vein was cannulated with an 8F introducer (Argon Medical Devices, Athens, Texas). Animals were exsanguinated and blood was collected in citrated triple blood donation bags (Teruflex; Terumo Medical Corp, Tokyo, Japan). Whole blood was centrifuged at 5000*g* for 9 minutes at 4°C, and plasma was removed by means of a plasma extractor (Baxter Healthcare, Deerfield, Illinois). Plasma was stored at –20°C for transport to a laboratory (HemCon Medical Technologies Inc, Portland, Oregon) for lyophilization. Pooled, sterile LP was returned to us and stored at room temperature for up to 1 month. Immediately before use, LP was reconstituted to its original volume with sterile water containing ascorbic acid for pH adjustment.

PLASMA CLOTTING FACTOR LEVEL MEASUREMENTS

Samples of plasma were analyzed for levels of fibrinogen, protein C, and antithrombin III, and factors II, V, VII, VIII, IX, X, XI, and XII by means of a coagulation system machine (BCS; Dade Behring Inc, Marburg, Germany) at the time of initial plasma preparation and after LP was fully reconstituted. Functional clotting assays for partial thromboplastin time (PTT) and prothrombin time (PT) were performed at the same 2 time points.

ANIMAL MODEL

The swine model used was a well-validated model of severe injury and hemorrhagic shock described by Cho et al[13] (**Figure 1**). Briefly, 32 juvenile Yorkshire crossbred swine (8 per group) underwent anesthesia, mechanical ventilation, and placement of invasive lines. All animals underwent femur fracture with a captive bolt gun (Karl Schermer Co, Ettlinger, Germany) to produce a comminuted long-bone fracture with severe overlying soft-tissue injury. After laparotomy, animals were cooled to 33°C with intraperitoneal isotonic sodium chloride solution and underwent controlled hemorrhage by removal of 60% of their estimated blood volume via a central line followed by 30 minutes of shock. Animals were infused with isotonic sodium chloride solution, 0.9%, at volumes 3 times the controlled hemorrhage volume to induce acidosis and coagulopathy. To mimic operative rebleeding and to produce an injury allowing measurement of blood loss after randomization to treatment, animals received a grade V liver injury followed by 30 seconds of uncontrolled hemorrhage. After the uncontrolled hemorrhage period, the liver was packed tightly with laparotomy sponges. Swine were randomized to receive either FFP, LP, FFP-PRBCs in a 1:1 ratio, or LP-PRBCs in a 1:1 ratio at 50 mL/min, with infusion of volumes equal to the blood removed during controlled hemorrhage. Resuscitation was initiated at the time of the liver packing. Animals were then monitored for 4 hours after resuscitation and subsequently chemically killed.

Hemodynamic data (heart rate and blood pressure) were recorded continuously throughout the procedure. Blood loss after liver injury was carefully recorded with the use of preweighed laparotomy sponges and a preweighed suction canister. Plasma samples were tested for PT, PTT, and lactate at baseline, after femur fracture, before controlled hemorrhage, before liver injury, and hourly for 4 hours after resuscitation with study fluid. To quantify levels of interleukin 6 (IL-6), IL-8, and tumor necrosis factor, plasma samples were collected before liver injury and 2 and 4 hours after administration of study fluids and quantified by commercially available enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minnesota).

STATISTICAL ANALYSIS

Data were analyzed with SPSS statistical software, version 16.0 (SPSS Inc, Chicago, Illinois). Variables were assessed for normal distribution. Normally distributed data were reported as means with standard deviations, and data that were not normally distributed were reported as medians with interquartile ranges. Comparisons between groups at the various time points were analyzed by means of independent *t* tests when the data were normally distributed and Kruskal-Wallis tests when the data were not normally distributed. Paired-samples *t* tests were used to compare same-group samples across various time points. Significance was denoted at $P < .05$.

RESULTS

EFFECT OF LYOPHILIZATION ON FACTOR FUNCTION

On average, clotting factor levels were decreased to 84% of their prelyophilization values (**Figure 2**). Compared with prelyophilization values, factor V retained 84% activity, factor VIII retained 84% activity, factor IX retained 100% activity, and antithrombin III retained 93%

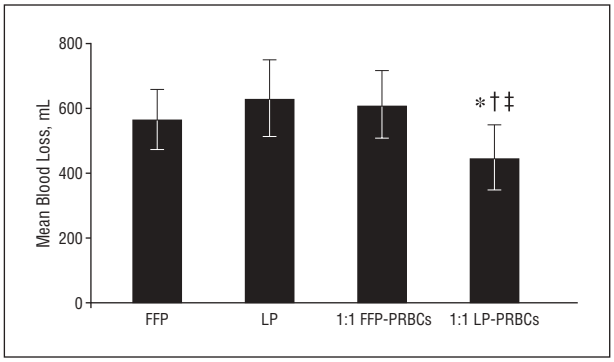

Figure 3. Total blood loss. Values are expressed as mean (SD). FFP indicates fresh frozen plasma; LP, lyophilized plasma; and PRBCs, packed red blood cells. *LP-PRBC group less than FFP group (447 mL vs 564 mL, *P*=.03). †LP-PRBC group less than LP group (447 mL vs 630 mL, *P*=.004). ‡LP-PRBC group less than FFP-PRBC group (447 mL vs 609 mL, *P*=.006).

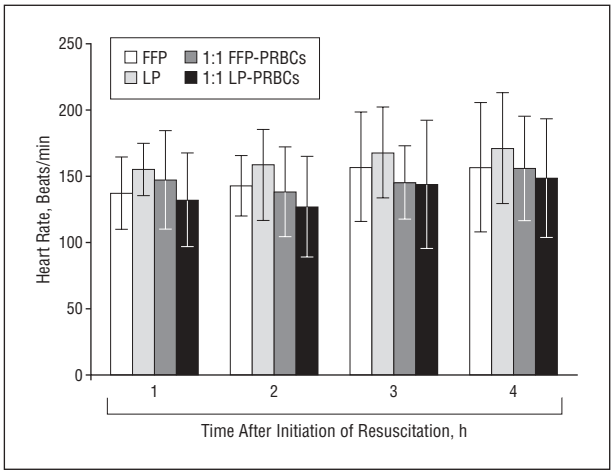

Figure 4. Heart rate. Values are expressed as mean (SD). FFP indicates fresh frozen plasma; LP, lyophilized plasma; and PRBCs, packed red blood cells. *P* values were not significant at all time points after initiation of resuscitation.

activity. The international normalized ratio was prolonged by 9% and the PTT was prolonged by 13% compared with prelyophilization values.

EFFECT OF DIFFERENT RESUSCITATION REGIMENS ON OUTCOMES

No pigs died before the end of the study in any group. The group that received 1:1 LP-PRBCs had significantly less blood loss than animals in other groups (**Figure 3**). There was no difference in blood loss among the other groups and no differences between the 4 resuscitation groups with respect to heart rate after resuscitation (**Figure 4**). The mean arterial pressure was significantly lower at various time points after resuscitation in the FFP group than the other 3 groups (**Figure 5**). The mean arterial pressure in the LP-PRBC group was significantly higher than that in the LP group at 3 hours after resuscitation.

Lactate levels and PT were similar in all groups at all 4 postresuscitation time points (**Figure 6** and **Figure 7**). The PTT in the FFP group was significantly lower than in the LP-PRBC and FFP-PRBC groups at all 4 time points after resuscitation (**Figure 8**). The PTT in the LP group was significantly lower than in the LP-PRBC and FFP-PRBC groups at variable time points after resuscitation.

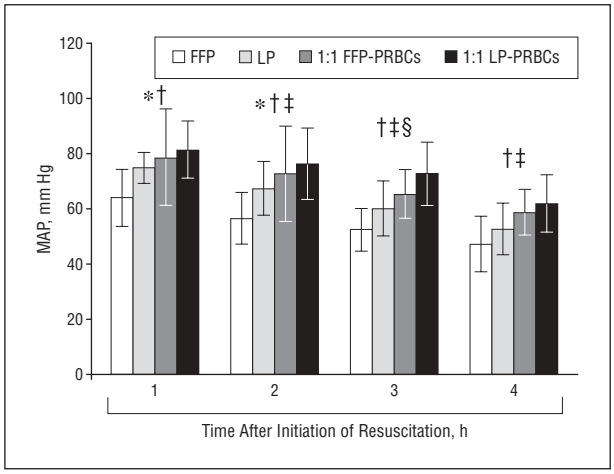

Figure 5. Mean arterial pressure (MAP). Values are expressed as mean (SD). FFP indicates fresh frozen plasma; LP, lyophilized plasma; and PRBCs, packed red blood cells. *LP group higher than FFP group. †LP-PRBC group higher than FFP group. ‡FFP-PRBC group higher than FFP group. §LP-PRBC group higher than LP group. (For all of these comparisons, $P<.05$.)

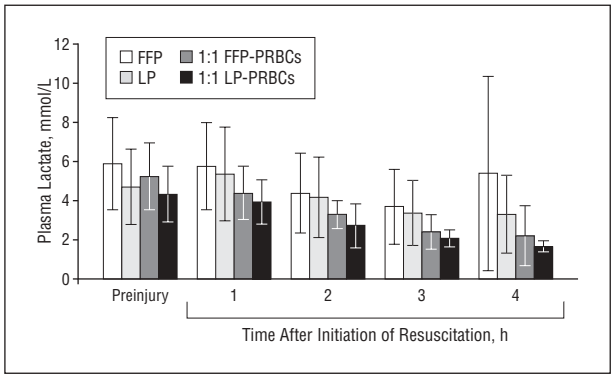

Figure 6. Plasma lactate levels. Values are expressed as mean (SD). FFP indicates fresh frozen plasma; LP, lyophilized plasma; and PRBCs, packed red blood cells. Plasma lactate levels were similar between all groups at all time points after initiation of resuscitation. To convert lactate to milligrams per deciliter, divide by 0.111.

There were no significant differences between LP and FFP or between the 1:1 groups with respect to IL-8 and tumor necrosis factor at any time point in the study. The IL-6 levels in the LP group were significantly less than in the FFP-PRBC group at 2 hours after injury and significantly less than in the FFP group at 4 hours after injury (**Figure 9**). The IL-6 levels in the LP-PRBC group were significantly less than in the FFP-PRBC group at 2 hours.

COMMENT

This study shows that the lyophilization process results in a modest reduction in clotting factor activity in vitro. Interestingly, there was no evidence of this reduction in the in vivo animal study. The study also shows that LP is as safe and effective as FFP for resuscitation after severe multisystem injury.

The first goal of this study was to characterize the effect of lyophilization and reconstitution of the plasma on coagulation factor activity. Variability in the makeup of LP was avoided primarily by pooling the plasma before lyophilization. On average, clotting factors were decreased

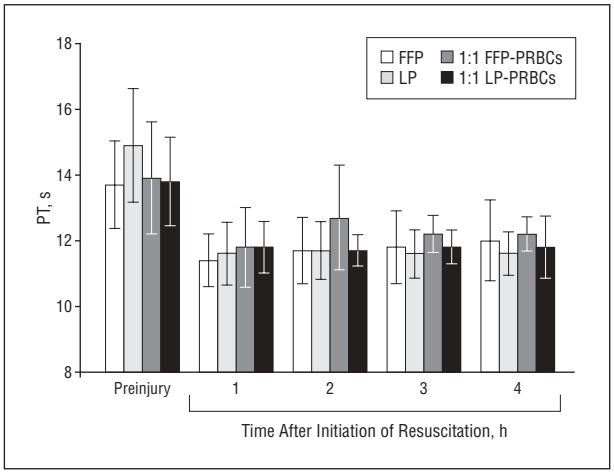

Figure 7. Prothrombin time (PT). Values are expressed as mean (SD). FFP indicates fresh frozen plasma; LP, lyophilized plasma; and PRBCs, packed red blood cells. Prothrombin time was similar between groups at all time points after initiation of resuscitation.

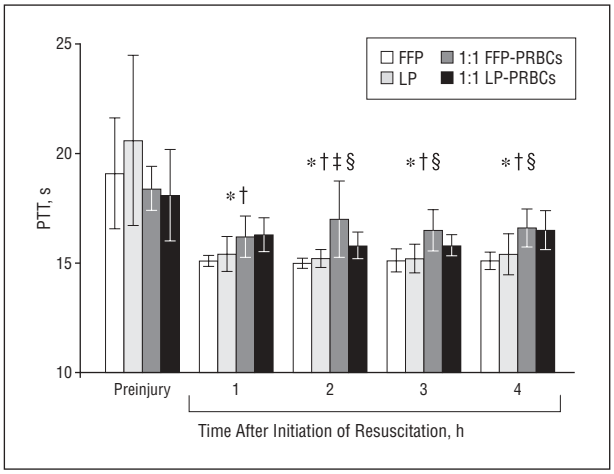

Figure 8. Partial thromboplastin time (PTT). Values are expressed as mean (SD). FFP indicates fresh frozen plasma; LP, lyophilized plasma; and PRBCs, packed red blood cells. *FFP group lower than LP-PRBC group. †FFP group lower than FFP-PRBC group. ‡LP group lower than LP-PRBC group. §LP group lower than FFP-PRBC group. (For all comparisons, $P<.05$.)

by 14%. This compares favorably with the standard 25% to 40% reduction caused by freezing and thawing FFP.[14,15] Industry standards for assessing the quality of FFP require the thawed FFP to maintain factor V, factor VIII, and antithrombin III levels of at least 70%.[14,15] Our study demonstrated that the LP retained much greater than the required 70%. In addition, the overall 14% reduction in clotting factor levels seemed to have only a minor effect on the functional clotting assays for PTT and PT. Even more important, LP was at least as effective as FFP in reversing the coagulopathy induced in the animal model; in all animals PT and PTT trended back toward normal after resuscitation, regardless of group. The finding of decreased blood loss in the group that received a 1:1 ratio of LP-PRBCs suggests that LP has an interaction with red blood cells that is not reflected in routine coagulation measures. We plan to better characterize this interaction in future studies.

Multiple studies have shown a correlation between elevated lactate levels and development of complications after trauma.[16-18] In our study, the groups had similar baseline lactate levels (after hemorrhage, shock, and acidosis) and the levels decreased and remained similar throughout the remaining 4 hours of observation. This finding supports the concept that LP is as effective as FFP for resuscitation. Interestingly, the groups resuscitated with a pure plasma product (either FFP or LP) did not have significantly higher lactate levels than the groups resuscitated with 1:1 ratios despite additional oxygen-carrying capacity in those groups.

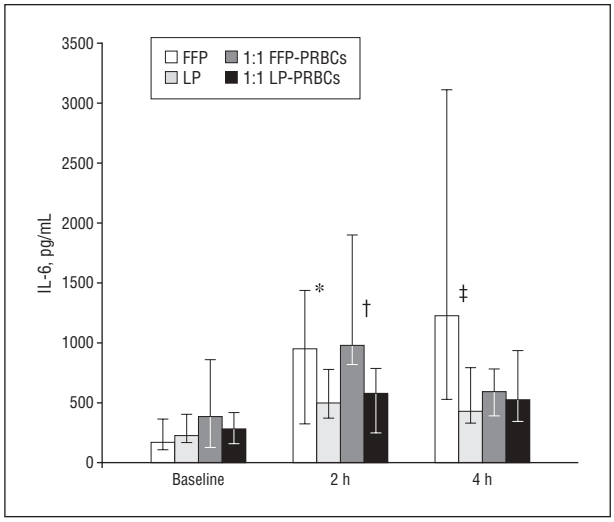

Figure 9. Interleukin 6 (IL-6). Values are expressed as medians with interquartile ranges. FFP indicates fresh frozen plasma; LP, lyophilized plasma; and PRBCs, packed red blood cells. *LP group less than FFP-PRBC group ($P=.02$). †LP-PRBC group less than FFP-PRBC group ($P=.009$). ‡LP group less than FFP group ($P=.049$).

Although this model is multisystem, well validated,[13] and representative of severe trauma and hemorrhagic shock, the volumes of transfused blood are not proportionally equivalent to those given in massive transfusions. The greatest benefits from transfusion with 1:1 FFP-PRBCs have been seen in patients who received massive transfusions.[2-10] In studies of patients receiving massive transfusions, the mean amount of blood received in 24 hours was 22 units of PRBCs.[5] The absence of massive transfusion in this study may explain why there were only minimal differences between the plasma groups and the 1:1 ratio groups.

The fact that resuscitation with LP resulted in decreased expression of the inflammatory cytokine IL-6 has some interesting implications. This is, to our knowledge, the first study to evaluate the effect of LP on posttraumatic inflammatory cytokines. Levels of IL-6, IL-8, and tumor necrosis factor have been found to be increased after trauma, and they are predictors of death,[19] systemic inflammatory response syndrome,[20] injury severity,[21-23] and multiorgan dysfunction.[24] One possible explanation for the blunted IL-6 response is that ascorbic acid was used to adjust the pH of the LP reconstitution fluid. Antioxidant levels are rapidly depleted in critical illness.[25] Ascorbic acid, acting as an antioxidant, has been shown to decrease inflammatory mediators,[26] reduce mortality,[27,28] and reduce multiorgan failure.[29]

The combined data on mortality, blood loss after liver injury, coagulation measures, and lactate demonstrate that LP is as effective as FFP for resuscitation after severe injury. Lyophilized plasma performed as well as or better than FFP in all of the foregoing areas.

Potential areas of concern with the LP include its ultimate shelf life, its performance when stored at extremes of temperature, and its potential for disease transmission. These important areas are currently under investigation through ongoing evaluations of the LP. Its shelf life appears to be quite long; preliminary studies demonstrated that LP retains factor activities comparable to those of thawed plasma when stored at 42°C for up to 9 months. Stability studies for a phase 1 clinical trial are currently in progress. The reconstituted product also appears to be stable at a broad range of temperatures. An initial investigation showed that the reconstituted LP demonstrated a stability profile similar to that of thawed FFP for up to 4 hours when stored between room temperature and 42°C and for up to 5 days when stored between 2°C and 8°C.

The infectious risk of LP is hypothesized to be similar to that of FFP. Exact determination of infectious risk has not been fully characterized because all human studies with LP have been conducted with the use of screened donor plasma purchased from a licensed blood bank. In addition, because some viral preparations are lyophilized to maintain stability, it is unlikely that the lyophilization process alone would change the infectious risk of the product. Treatment of LP with a solvent detergent to reduce infectious risk is a potential option, but a combination of a loss of some coagulation function with reports of thromboembolic events and deaths following transfusion of solvent detergent–treated plasma has resulted in the withdrawal of solvent detergent–treated plasma from the US market.[30]

Although our swine model is designed as a surrogate for human injury and resuscitation, the applicability of our study findings to human situations has not been fully evaluated. Initial studies comparing factor activities and coagulation assays between swine LP and swine FFP have demonstrated overall similarity to human LP and human FFP. The species-specific differences in factor activities will require ongoing investigation to ensure full safety and efficacy. Our future investigations will include a comprehensive evaluation of the effects of the lyophilization process on coagulation properties of the LP. We will do this by characterizing the coagulation properties of freshly prepared plasma and comparing them with the properties of reconstituted LP. We will also perform comprehensive coagulation evaluations on swine receiving LP.

The findings of this study suggest that LP has great promise as a resuscitation fluid in the combat and prehospital settings as well as in the hospital. The 2001 Fluid Resuscitation in Combat conference in Toronto, Ontario, Canada, with support from the Office of Naval Research, described the ideal resuscitation fluid as able to "provide rapid volume expansion with restoration of tissue perfusion so as to prevent or delay the onset of hypovolemic shock." In addition, the fluid should be capable of "dampening an overzealous immune response," and "given the unique . . . medical constraints of the far-forward military environment . . . fluid volume [should] be minimized."[31(pS10)] Fresh frozen plasma, because of the strict limitations for storage and preparation, is impractical in satisfying these requirements. In light of the fact that lyophilized plasma can be stored at room temperature, easily transported, and quickly reconstituted, it appears to be an ideal resuscitation fluid for all settings.

Accepted for Publication: May 26, 2009.
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Financial Disclosure: None reported.
Funding/Support: This study was supported by the US Army Medical Research Acquisition Activity Award No. W81XWH-04-1-0104.
Previous Presentation: This paper was presented at the 80th Annual Meeting of the Pacific Coast Surgical Association; February 15, 2009; San Francisco, California; and is published after peer review and revision. The discussions that follow this article are based on the originally submitted manuscript and not the revised manuscript.

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DISCUSSION

David Hoyt, MD, Orange, California: About 76 years ago, a surgeon in North Carolina separated plasma from blood and demonstrated it could resuscitate a civilian stab wound to the heart. Plasma dominated World War II resuscitation because it could be preserved in hostile environments and offered superiority to crystalloid.

This began a long debate that ultimately led to the reversal of our thinking regarding plasma resuscitation. During the intervening 75 years, we have had continued discussion about the best way to use component blood therapy. Following Vietnam, John Collins suggested that incremental replacement of specific deficits with component therapy was the best way to treat posttraumatic coagulopathy. This started a series of debates that intensified over the last 10 years regarding whether to give platelets first or FFP first, and whether to always base this decision on a laboratory test.

During the Iraq conflict and the use of an infrastructure supported by the Institute for Surgical Research to study early resuscitation by Drs Holcomb and Schreiber and many others, we have learned that early reconstitution of plasma and red blood cells toward the equivalent of whole blood is superior in the most severely injured patients. The present study is a breakthrough in the availability of a stable source of FFP that can be reconstituted in a hostile environment or during routine transfusion without the delay of thawing.

A recent consensus conference suggested that most major trauma centers have focused on the need to reconstitute red blood cells with FFP as having great potential to save lives. Studies proving this are in the process of design. The availability of a product that makes this easier and appears equivalent in preclinical models is very exciting. These studies show that the lyophilization process decreases clotting activity by only 14%, and the use of this product seems to be equivalent to actual FFP. I have several questions:

1. In the manuscript, you suggest that the shelf life of this product has been tested to 1 month. What do we think the ultimate shelf life will be?
2. You report loss of activity of 14% with lyophilization. Would this be similar with human plasma? Are there data to suggest what activity loss will be? Will this be compatible with translation into a human study?
3. Do you see this as replacing current practice with FFP by offering a manufactured product that would relieve blood banks from the freezing and thawing logistical problems? If so, is this going to be expensive?
4. Finally, does the processing and lyophilization process reduce the infectious risk relative to FFP?

This is an important study and will provide the preclinical basis for a clinical trial.

Dr Schreiber: Regarding issues of half-life and functionality of this product compared with human plasma, we are partnering with HemeCon, which is an industrial organization, to develop this product and these studies are currently being done. What has been found with the human product is essentially the same decrease in factor activity—about 14% to 16% decrease in activity immediately after lyophilization, but after that the product stabilizes. We have data that show that the product remains the same over about a 3-month period, and we don't know how long this product will be functional, but we are estimating currently that it may be good for up to 1 or 2 years and can be maintained at room temperature. So you are going to have a powder you can keep anywhere, including on an ambulance, in Iraq or Afghanistan, and you just put a little water in it and you have some plasma. It seems to be pretty stable for long periods of time.

The product will go beyond replacing FFP because it can be made better than FFP. We are entering into the next phases of our study, which are looking at 3 primary things. The first is how to reconstitute this LP. Currently it is being reconstituted with water that contains ascorbic acid to its original volume. One of the things that is being looked at is reconstituting it with less volume, creating hypertonic plasma. This is advantageous for several reasons, but primarily, in the military all of the fluid that is given has to be carried on the backs of our soldiers. So if we can reconstitute this material with less volume, it's going to be even more functional than it currently is out in the battlefield.

The second thing is what would be the best reconstitution fluid. We are currently using water. Maybe we should use hypertonic saline, lactated Ringer solution, normal saline [isotonic sodium chloride solution], or some other fluid, and we can make it better than the original product.

And finally, we can add whatever we want to this product. Currently it has ascorbic acid simply to correct the pH of the alkalotic solution when it is reconstituted. We can adjust the amount of ascorbic acid, add other antioxidants, and make it much better than the current plasma, and the results that show decreased inflammation are very promising.

The final question was concerning infectious diseases. Does the lyophilization process prevent transmission of viral diseases? We don't know. It may not, because viruses can be lyophilized and still maintain their function. So this is also being studied by putting viruses into the plasma, lyophilizing it, and then checking for viral activity. But we don't know the results at this time.

Financial Disclosure: None reported.