Hemostatic characteristics of thawed, pooled cryoprecipitate stored for 35 days at refrigerated and room temperatures

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BACKGROUND: Cryoprecipitate's shelf life is limited due to concerns over decreased clotting factor activity and contamination with extended storage. Hemostatic characteristics of thawed cryoprecipitate stored up to 35 days at refrigerated and room temperatures were assessed.

STUDY DESIGN AND METHODS: Pooled cryoprecipitate was thawed and aliquoted for storage at 1–6°C or 21–24°C. Samples were tested immediately after thawing and at 4 h, 24 h, 72 h, and weekly for 35 days. At each time point fibrinogen, factor VIII (FVIII), and von Willebrand factor (vWF) were assessed. Thrombin generation and rotational thromboelastometry (ROTEM) were also performed. Further, packed red cells, platelet concentrates, frozen plasma, and stored cryoprecipitate were combined (1:1:1:1) to simulate massive transfusion and analyzed by ROTEM. Day 35 samples were cultured for bacterial contamination.

RESULTS: Precipitation was observed in refrigerated samples; however, these aggregates were easily resuspended upon warming in a 37°C water bath. No significant changes were observed in fibrinogen concentration or ROTEM at either temperature. FVIII and vWF declined significantly during storage. vWF, clot time, and thrombin generation were significantly better preserved with refrigeration. With simulated massive transfusion, fibrinogen function remained at or above the established range for whole blood at both storage temperatures. Bacterial contamination was not observed in cold stored or room temperature cryoprecipitate.

CONCLUSION: The fibrinogen concentration and function of cryoprecipitate at extended storage durations are adequate for fibrinogen replacement in critical bleeding. These results support extension of the shelf life of cryoprecipitate when used for fibrinogen replacement.

Despite advances in management of trauma patients, hemorrhagic shock remains the leading cause of preventable death after traumatic injury. Trauma-induced coagulopathy (TIC) is a frequent complication of severe trauma that is associated with increased transfusions, post-traumatic organ failure, and mortality.[1] Accumulating evidence supports the central role of hypofibrinogenemia, fibrinolysis, and dysfibrinogenemia in TIC.[2,3] Trauma patients have decreased levels of fibrinogen on hospital admission, and low fibrinogen is associated with increased mortality in these patients.[4–7] Further, fibrinogen falls to critical levels earlier in massive hemorrhage than any other factor, and several retrospective studies have suggested mortality is improved in patients who receive fibrinogen replacement as part of their resuscitation.[5,6,8–10]

Fibrinogen can be replaced with plasma, fibrinogen concentrates (FCs), or cryoprecipitate; however, fibrinogen concentrations in plasma are inadequate to overcome TIC.[11] Cryoprecipitate is the most commonly used fibrinogen replacement product in the United States, United Kingdom and worldwide. FCs have replaced cryoprecipitate in many European countries, however, primarily because they are virally inactivated, have a standardized dose, and are easier to store and administer.[12] FCs are much more expensive

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than cryoprecipitate and are difficult to obtain or unavailable in some countries and in austere/operational environments.[13] Further, there is a lack of high-quality evidence supporting fibrinogen replacement with one product over the other in terms of safety or efficacy.[14]

Cryoprecipitate was first introduced to replace factor VIII (FVIII) in hemophilia A, but today it is primarily used for fibrinogen replacement in trauma and critical bleeding. Cryoprecipitate is derived from the precipitate formed when fresh frozen plasma is thawed at 1–6°C. After centrifugation of the thawed plasma and removal of the supernatant, the cryoprecipitate is re-suspended and stored frozen for up to 12 months. In addition to fibrinogen, cryoprecipitate contains a high concentration of FVIII, von Willebrand factor (vWF), FXIII, and fibronectin. It is frequently pooled from five single plasma units and, in practice, administered as 10 units per adult dose.[15] Prior to transfusion, cryoprecipitate is thawed at 30–37°C. Current guidance from the US Food and Drug Administration (FDA) and clinical practice guidelines mandates that thawed cryoprecipitate be stored at room temperature and transfused within 4 hours of thawing or within 6 hours if pre-pooled in a closed system prior to freezing.[4] A reactive approach to fibrinogen replacement is also recommended, so cryoprecipitate transfusion is generally initiated only after a fibrinogen deficiency has been identified on laboratory evaluation.[16] These recommendations severely limit the usage of cryoprecipitate for fibrinogen replacement.

Interest in examining the role of fibrinogen replacement early in the management of massive hemorrhage has grown in recent years. The major hurdles to achieving timely transfusion of cryoprecipitate in a clinical trial setting, or in clinical practice for that matter, are the required thawing time and narrow shelf life. The PROMMTT study observed a 2.7-hour median time to delivery of cryoprecipitate at ten level-1 trauma centers in the United States: of those patients who died of hemorrhage within 6 hours of admission, 72% did not receive cryoprecipitate transfusion during resuscitation.[17] The CRYOSTAT study assessed the feasibility of early administration of cryoprecipitate in bleeding trauma patients and found that the median time from admission to product transfusion was 60 minutes, which may be far too long for patients presenting with major bleeding.[18] Transfusion of cryoprecipitate very early during resuscitation may improve the mortality of traumatic hemorrhage; however, current guidelines for storage and use of cryoprecipitate make its evaluation in a clinical trial challenging.[5,6]

One concern with biological products is that storage at room temperature for an extended duration increases the risk of bacterial growth. Refrigeration is an accepted strategy for reducing the risk of contamination of blood products, including thawed plasma; however, the effects of refrigeration on the quality of cryoprecipitate at extended storage durations has not been evaluated. The aim of this study was to evaluate the hemostatic characteristics of thawed, pooled cryoprecipitate for 35 days (the current limit of refrigerator-stored whole blood) to assess the feasibility of increasing shelf life. Additionally, we compared the hemostatic function of cold-stored and room-temperature stored cryoprecipitate.

MATERIALS AND METHODS

Cryoprecipitate was purchased in six-donor frozen pools (Type O and Type A donor pools, n = 4 each) from South Texas Blood and Tissue Center along with pooled frozen cryoprecipitate-poor plasma (CPP). Cryoprecipitate was thawed at 37°C on the initial day of testing and distributed into 6 mL aliquots under sterile conditions for usage at each subsequent time point. Stored samples were maintained either at room temperature (21–24°C) or refrigeration (1–6°C) in low humidity conditions until the day of testing.

Coagulation factors

Cryoprecipitate was diluted 1:10 with phosphate-buffered saline and analyzed for fibrinogen (STA-Fibrinogen 5, Stago) and Factor VIII (STA-Deficient VIII, Stago) content with the STA-R Evolution clinical hemostasis analyzer (Stago) according to the manufacturer's instructions. Activity of vWF was determined by mixing diluted cryoprecipitate with freeze-dried platelets from the ristocetin cofactor assay kit (Chrono-Log Corporation) in the Chrono-Log 700 device (Chrono-Log) and measuring aggregation by transmission according to the manufacturer's specifications.

Thrombin generation

Stored cryoprecipitate was combined with CPP at the original ratio (1:10) to generate a reconstituted plasma product. Thrombin generation was measured using a calibrated automated thrombogram (CAT) assay by mixing 80 μL of plasma with 20 μL of PPP-Low reagent (Stago) containing 1 pM tissue factor and 4 μM phospholipids, incubating for 10 minutes (37°C), and then mixing with 20 μL Z-Gly-Gly-Arg-AMC fluorogenic substrate and calcium chloride. Thrombograms were generated using the Fluoroskan Ascent FL (Thermo Fisher Scientific) and Thrombinoscope software (Thrombinoscope BV). Parameters of interest included lagtime, thrombin peak, endogenous thrombin potential (ETP), and time-to-peak (ttPeak). Triplicate samples were measured alongside additional samples run with thrombin calibrator as an internal control.

Rotational thromboelastometry

The coagulation function of reconstituted plasma (1:10 cryoprecipitate: CPP) was measured with rotational thromboelastometry (ROTEM delta, Instrumentation Laboratory). Reagents for the EXTEM test (tissue factor pathway activation, 40 μL total) were mixed with 300 μL of sample according to the manufacturer's instructions and allowed to clot for 2 hours.

Simulated massive transfusion protocol

A simulation of the products delivered in a massive transfusion protocol (MTP) was created by combining packed red blood cells (pRBCs), platelet concentrates, fresh frozen plasma (FFP), and stored cryoprecipitate at a ratio of 1:1:1:1. For these samples, whole blood (WB) was collected in citrate–phosphate-dextrose from healthy donors under an approved institutional standard operating procedure at the US Army Institute of Surgical Research Blood Bank and processed for red cells through centrifugation and addition of AS-5 preservative solution by American Association of Blood Banks (AABB)-approved techniques. Concentrated platelets were obtained from WB which was collected in acid citrate dextrose (ACD) vacutainers (Becton Dickinson). This WB was centrifuged at 200-g for 10 minutes to separate platelet-rich plasma which was subsequently pelleted using 1 μM prostaglandin I2 (Sigma-Aldrich) to prevent aggregation of the platelets; these were resuspended in ACD plasma at a concentration of 1000 platelets/nL to approximate the normal count for apheresis collection of platelets. FFP was obtained from aliquots of type-matched apheresis-collected plasma, which were frozen immediately after collection.

Simulated MTP samples were analyzed by ROTEM as described earlier with both EXTEM (for global hemostatic function) and FIBTEM (to isolate the fibrinogen contribution to clot strength) assays according to the manufacturer's protocols.

Cultures

As a quality-control measure at the end of the study, room temperature and refrigerated samples of cryoprecipitate were assessed for bacterial contamination at 35 days. Samples were inoculated to both agar and broth media. Tryptic soy agar/broth were utilized for isolation of gram-positive contaminants and LB-Lennox agar/broth were utilized for isolation of gram-negative contaminants. Samples inoculated to agar were incubated in a CO_2 incubator (5%) at 37°C and monitored for 72 hours. Broth cultures were assessed for turbidity and subcultured daily to appropriate agar media to assess bacterial growth.

Statistical analysis

Data collection and statistical analyses were achieved with Excel 2010 (Microsoft) and Prism 7.01 (GraphPad). Two-way analysis of variance tests using Dunnett's multiple comparison correction were used to measure differences between each time point and the baseline day 0 measurements; Sidak's multiple comparison test was used to make comparisons between storage at 4 °C and 22 °C at each time point.

RESULTS

Because no significant differences were observed in the hemostatic properties of group A and group O samples, these were combined for data analysis with the mean results from all eight cryoprecipitate pools summarized in each figure. Precipitation was observed in the refrigerated samples, but these aggregates were easily re-suspended upon warming in a 37°C water bath for 5 minutes and mixing with vortex immediately prior to testing.

Coagulation factors

Fibrinogen concentration was stable for the first 2 weeks (Fig. 1); although a decrease was observed beginning at 21 days, it was only significant in the room temperature samples on day 35 ($p = 0.006$ vs. day 0). Additionally, the fibrinogen concentration of refrigerated and room temperature cryoprecipitate did not differ significantly at any time point.

After thawing, FVIII activity declined progressively through the end of the study with a significant drop by

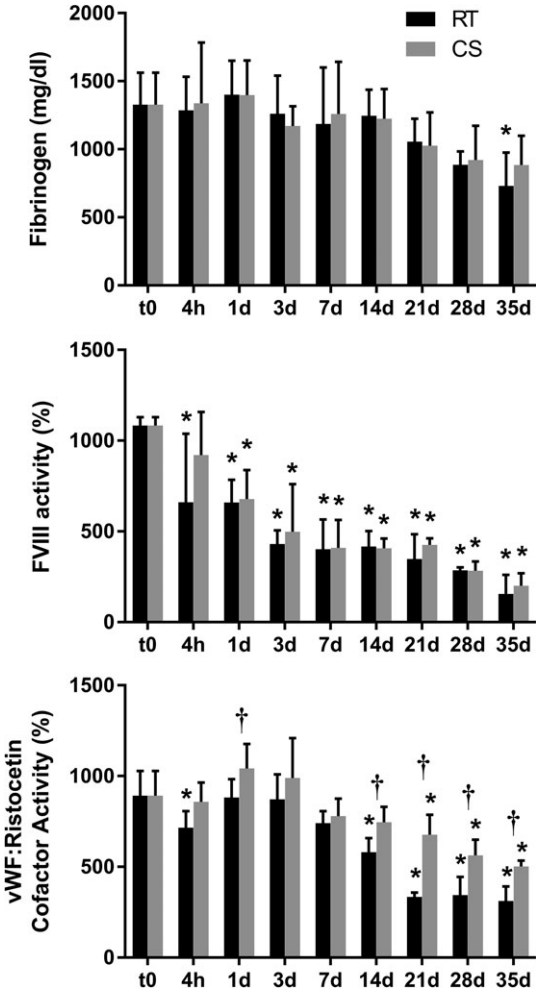

Fig. 1. Fibrinogen concentration was well-maintained to 35 days, while FVIII activity declined rapidly and von Willebrand factor activity was significantly diminished at 14 days and beyond. * $p < 0.05$ versus t0 measurement. † $p < 0.05$ for differences between room temperature (RT) and cold-stored (CS).

4 hours in room temperature (p = 0.002) and 24 hours in cold-stored (p = 0.0005). Differences between room temperature and refrigerated samples were not significant at any time point.

vWF activity increased through the 24-hour evaluation before declining gradually through the end of the study reaching significance at 14 days in the room temperature samples (p < 0.0001) and 21 days in the refrigerated samples (p = 0.002). The peak vWF activity (occurring at the 24-hour time point) was higher in the refrigerated cryoprecipitate (1041% vs 881%, p = 0.038).

Thrombin generation

In the room temperature cryoprecipitate, ETP and peak thrombin declined after thawing (Fig. 2) reaching significance at 72 hours (ETP, p = .021; peak thrombin, p = .011); ttPeak increased gradually and became significant at 28 days (p = .022).

In the refrigerated cryoprecipitate, ETP and peak thrombin increased between thawing and 72 hours before declining to a significantly lower level at 21 days (33% decline in mean ETP by day 21, p = 0.003; 46% decline in mean peak thrombin, p = 0.021). The ttPeak decreased significantly between thawing and 72 hours (p = 0.002) and increased gradually thereafter, but there was no significant increase from baseline at 35 days (p = 0.97).

Compared to room temperature-stored cryoprecipitate, refrigerated cryoprecipitate had significantly higher ETP (at 72 h, 14 d, 21 d, and 35 d) and peak thrombin (at 24 h and 72 h); ttPeak was significantly lower in refrigerated samples from days 14 through 35.

Plasma coagulation function

For each sample, there were no significant changes versus baseline or differences between room temperature and cold-stored cryoprecipitate in any of the major EXTEM parameters (Fig. 3), including clotting time (CT), maximum clot firmness (MCF, a measure of clot strength), alpha angle (a measure of clot rate), or lysis index at 30 minutes (LI30, the degree of clot lysis 30 minutes after MCF has been reached).

Simulated massive transfusion protocol

On EXTEM (Fig. 4), the CT increased after thawing in the room temperature cryoprecipitate with significance reached at the day 7 evaluation (p = 0.003 vs. day 0). The CT of refrigerated cryoprecipitate actually decreased over the first 72 hours (p = 0.0004), increasing thereafter, though never reaching statistical significance. The CT of refrigerated samples was better preserved at every time point after baseline. MCF and alpha angle had slight but primarily insignificant decreases over storage in both samples, although the alpha angle was decreased on day 35 in the refrigerated sample (p = 0.009 vs. day 0, p = 0.003 vs. room temperature on day 35). There were small but significant declines from baseline in LI30 at 7 days through 21 days for room temperature, but

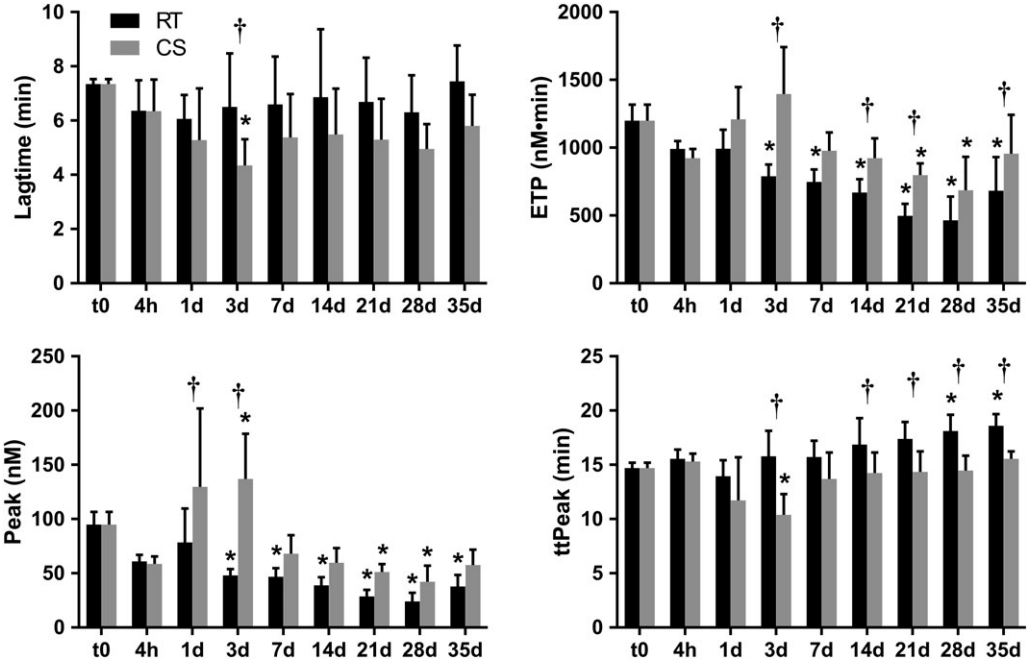

Fig. 2. Thrombin generation parameters show that lagtime declined in cold-stored (CS) samples, significantly only on day 3; while endogenous thrombin potential (ETP) and peak thrombin increased out to day 3 before declining through the remaining storage time. ETP and peak thrombin of room-temperature (RT) samples declined throughout storage, significant by day 3. Time-to-peak (ttPeak) was shown to increase in RT samples by day 28. * p < 0.05 versus t0 measurement. † p < 0.05 for differences between RT and CS.

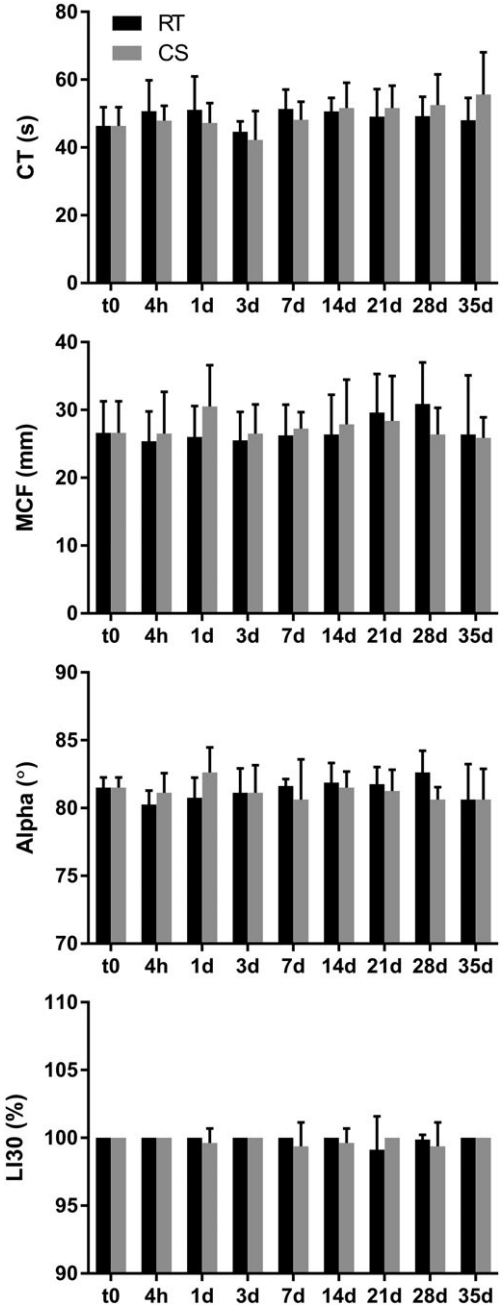

Fig. 3. Room temperature (RT) and cold-stored (CS) cryoprecipitate was diluted in cryoprecipitate-poor plasma at original ratios (1:10) then activated with EXTEM reagents. No significant changes were observed in any of the coagulation parameters measured by ROTEM.

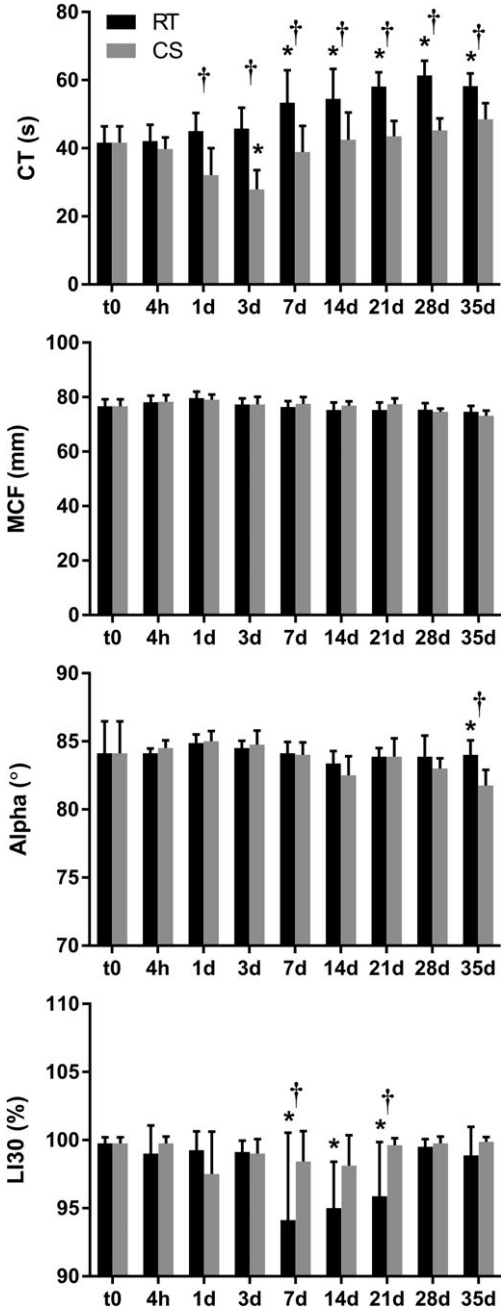

Fig. 4. A simulated massive transfusion protocol of packed red blood cells, platelet concentrates, fresh frozen plasma, and stored cryoprecipitate (1:1:1:1 mixture) was activated with EXTEM reagents. Significant increases in clotting time (CT) were observed for room temperature (RT)-stored cryoprecipitate samples, while maximum clot firmness (MCF) and alpha angle were maintained for the majority of storage. LI30 in RT-stored cryoprecipitate samples dipped between days 7 and 21 before returning to near-baseline measurements. * $p < 0.05$ versus t0 measurement. † $p < 0.05$ for differences between RT and cold-stored (CS).

LI30 increased thereafter with no significant differences at subsequent time points. The results of all measured parameters fell within or exceeded the normal values established for EXTEM of WB.

Like EXTEM, the CT on FIBTEM (Fig. 5) increased in room temperature cryoprecipitate after thawing reaching significance at 28 days whereas the CT of refrigerated cryoprecipitate decreased for 72 hours before increasing but these changes were not significant. Again, the difference in

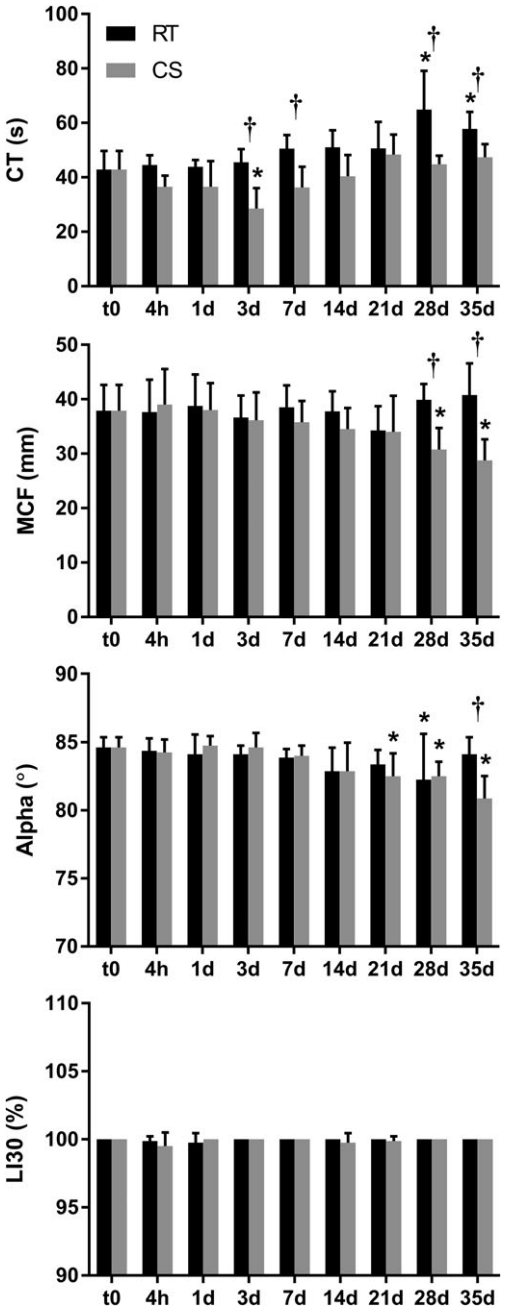

Fig. 5. A simulated massive transfusion protocol of packed red blood cells, platelet concentrates, fresh frozen plasma, and stored cryoprecipitate (1:1:1:1 mixture) was activated with FIBTEM reagents to inactivate the platelets and view the plasma/fibrinogen contribution to clot formation. Significant increases in clotting time (CT) were observed for room temperature (RT)-stored cryoprecipitate samples by day 14. Maximum clot firmness (MCF) and alpha angle declined in cold-stored (CS) samples on day 28 and 35. * $p < 0.05$ versus t0 measurement. † $p < 0.05$ for differences between RT and CS.

CT between room temperature and refrigerated samples was significant beginning at 72 hours. The MCF of room temperature cryoprecipitate did not change significantly from baseline; a small but significant decrease in MCF was seen in refrigerated cryoprecipitate at 28 and 35 days. Alpha angle declined after thawing reaching significance at 28 days in room temperature and 21 days in refrigerated cryoprecipitate. The CT and MCF results fell within or exceeded the reference values for FIBTEM of whole blood; the FIBTEM range for alpha angle has not been established.

Cultures

Colonies were detected 48 hours after plating on two of the four plates prepared directly from room temperature cryoprecipitate on day 35; no growth was detected in any of the broth cultures prepared from the same samples. At no point was bacterial contamination ever detected in the refrigerated cryoprecipitate samples.

DISCUSSION

Although cryoprecipitate was first introduced as a treatment for hemophilia A and other bleeding disorders, it has largely been replaced by coagulation factor concentrates and recombinant proteins for these indications.[19] In fact, clinical practice guidelines recommend against using cryoprecipitate for replacement of FVIII or vWF unless factor-specific products are unavailable.[4] Today, the primary use of cryoprecipitate is as a concentrated source of fibrinogen in acquired coagulopathy related to trauma, obstetric hemorrhage, liver transplant, and disseminated intravascular coagulopathy.[20]

The current FDA and AABB standards for cryoprecipitate were adopted to ensure adequate FVIII levels for treatment of hemophilia A, and have remained largely unchanged since their formulation in 1977. Since cryoprecipitate is now primarily used for fibrinogen replacement in critical bleeding, the current guidelines may be overly restrictive and may contribute to decreased or delayed utilization and product waste. Previously published work has demonstrated that fibrinogen concentration is maintained in cryoprecipitate stored at room temperatures for up to 5 days.[21–23] Recently, Green and colleagues showed that the function of fibrinogen, as measured by thrombin potential and ROTEM, in thawed cryoprecipitate stored at room temperature for 72 hours did not differ significantly from baseline.[21]

Here, we conducted the most extensive evaluation of the hemostatic properties of cryoprecipitate at extended storage durations to date. Fibrinogen concentration is not significantly changed with storage for 4 weeks at room temperature or 5 weeks in the refrigerator. In a reconstructed plasma consisting of stored cryoprecipitate diluted with CPP, the preservation of MCF, alpha angle, and LI30 on EXTEM suggests that the gradual decline in fibrinogen observed after 14 days may not be functionally or clinically relevant. There are no established ROTEM reference ranges for cryoprecipitate or plasma; however, both room temperature

and refrigerated cryoprecipitate remained within or above the ranges established for WB throughout the study during massive transfusion simulations. This suggests that even cryoprecipitate stored for 35 days would contribute to increasing the fibrinogen concentration and mitigate coagulopathy in trauma patients, and our simulated massive transfusion studies suggest that refrigerated cryoprecipitate would still have acceptable efficacy in the critically bleeding patient as part of a transfusion bundle.

Extended storage of blood products at room temperature raises the risk of bacterial contamination, and refrigeration is generally recognized to minimize contamination risk. Current AABB standards allow FFP to be used up to 5 days of storage after thawing in the refrigerator, and this practice is supported by several studies showing sterility is maintained with refrigeration.[24,25] There are very few studies evaluating contamination of cryoprecipitate at extended storage durations. Soundar and coauthors found no detectable bacterial growth in cryoprecipitate stored at ambient temperature for 6 hours then refrigerated until 24 hours.[26] Our studies showed no contamination over the 35 days of storage, but that suggests only that sterility was successfully maintained during collection. Inoculation studies would be necessary to prove that refrigeration provided a sufficient retardation of bacterial growth over 35 days.

Cryoprecipitate is not intended to replace FVIII or vWF in critical bleeding; nevertheless, we characterized these factors as well. As previously reported, FVIII levels decline precipitously after thawing, and vWF increases over the first 24 hours before declining at a much slower rate.[21,23,27] vWF activity was better maintained in refrigerated cryoprecipitate. Interestingly, the functional assays relating to thrombin generation and clot initiation were also better preserved in the refrigerated cryoprecipitate. Because the CT parameter of MTP simulation room-temperature samples was significantly prolonged, whereas the CT in cryoprecipitate + CPP samples was not significantly different, there is likely a platelet-mediated effect resulting from the observed changes to vWF activity in room-temperature samples at later time points. FXIII is important for crosslinking of fibrin and clot stabilization, but the amount of FXIII required to improve coagulation in TIC is poorly understood. There is evidence that replacement of fibrinogen and FXIII improves coagulation better than replacing either factor alone.[28] However, there is no standard for FXIII levels in cryoprecipitate, and we did not assess FXIII levels directly, a limitation of this study. The LI30 was not significantly changed between the beginning and end of the study, which suggests that clot stability and FXIII (indirectly) were not affected by extended storage. The changes in EXTEM LI30 observed at days 7–21 during simulated massive transfusion may have been platelet-driven given the normal corresponding FIBTEM LI30.

This study and others cited demonstrate sufficient evidence to increase the shelf life of cryoprecipitate, but absolute shelf life and proper storage temperature remain up for debate. Our results show that refrigerated cryoprecipitate could safely be extended to at least 14 days, but even at 35 days there is acceptable efficacy in emergency situations or resource-constrained environments. As an intermediate step, making a very conservative extension to 24 hours' storage at room temperature or 5 days if refrigerated seems practical. As written, the guidelines may impede timely administration of cryoprecipitate in critical bleeding and contribute to challenges in producing research aimed at improving trauma outcomes. An extension of the shelf life of cryoprecipitate, when used for fibrinogen replacement, would dramatically improve product delivery, decrease waste, and improve the feasibility of conducting high-quality prospective studies.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1: Thrombin generation curves of a representative sample of cold stored (CS) and room temperature (RT) cryoprecipitate at extended storage durations. In RT cryoprecipitate, thrombin generation declined after thawing reaching significance at 72 hours (endogenous thrombin potential [ETP], $p = 0.021$; peak thrombin, $p = 0.011$). In CS cryoprecipitate, thrombin generation increased between thawing and 72 hours before declining to a significantly lower level at 21 days (33% decline in mean ETP by day 21, $p = 0.003$; 46% decline in mean peak thrombin, $p = 0.021$).