Published in final edited form as:

J Surg Res. 2017 November ; 219: 145–150. doi:10.1016/j.jss.2017.05.030.

14-Day Thawed Plasma Retains Clot Enhancing Properties and Inhibits tPA-induced Fibrinolysis

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Abstract

Background—Plasma-first resuscitation attenuates trauma-induced-coagulopathy (TIC), however, the logistics of plasma-first resuscitation require thawed plasma (TP) be readily available due to the obligatory thawing time of FFP. The current standard is storage of TP for up to 5 days at 4°C, based on factor levels at outdate, for use in patients at risk for TIC, but there remains a 2.2% outdated wastage rate. However, the multitude of plasma proteins in attenuating TIC remain unknown. *We hypothesize that TP retains the ability to enhance clotting and reduce tPA-induced fibrinolysis at 14 days storage.*

Methods—FFP was thawed and stored at 4°C at the following intervals: 14, 10, 7, 5, 3, and 1-day prior to the experiment. Healthy volunteers underwent blood draws followed by 50% dilution with above TP stored intervals as well as FFP, normal saline (NS), albumin, and whole blood (WB) control. Samples underwent tPA-modified (75ng/ml) thrombelastography (TEG) with analysis of R-time, angle, maximum amplitude (MA), and LY30.

Results—TEG properties did not change significantly over the thawed storage. 14-day TP retained the ability to inhibit tPA-induced hyperfibrinolysis (median LY30% 9.6%) similar to FFP (5.6%), WB (14.6%) and superior to albumin (59.3%) and NS (58.1%). 14-day TP also retained

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Presented at Academic Surgical Congress; Las Vegas, NV, February 2017

Author Contribution: B.R.H., E.E.M., H.B.M., G.S., G.R.N., C.C.S. designed the experiment. B.R.H. and A.S. performed the analysis of the data. G.S., R.S., G.R.N., B.R.H. performed the acquisition of the data. B.R.H. wrote the manuscript which all authors critically revised for important intellectual content. All authors approved the final version and agreed to be accountable for all aspects of the work.

Disclosure: The authors do not have any other disclosures.

faster clot formation (median angle, 66.2°) and superior clot strength (MA, 61.5mm) to albumin (34.8°, 21.6mm) and NS (41.6°, 32.2mm).

Conclusion—TP plasma stored for 14 days retains clot enhancing ability and resistance to clot degradation similar to FFP. A clinical trial is needed to validate these *in vitro* results.

Keywords

Thawed plasma; fresh frozen plasma; hyperfibrinolysis; plasma transfusion; trauma-induced-coagulopathy; thrombelastography

Introduction

Hyperfibrinolysis is a lethal component of trauma-induced-coagulopathy (TIC), with an associated mortality approaching 50% from uncontrolled blood loss.^{1,2} Emerging *in vitro* and animal studies support the use of plasma-first resuscitation in the correction of hyperfibrinolysis and TIC,^{3,4} however, the logistic challenges of early plasma administration are well-documented,⁵ including transport, storage, and standard thawing time (30-minutes at 37°C). These logistic problems have led to pre-treatment thawing of plasma and storage at 4°C.

The current standard for TP storage is 4°C for up to 5 days to be used in situations requiring emergent administration.⁶ Even with the increased use of TP (30.4% of all plasma used in the United States), there remains a 2.2% outdated wastage rate in the United States, accounting for 129,000 units of plasma wasted each year (\$7.2 million).⁷ This 5-day lifespan of TP prevents smaller hospitals from having plasma readily available. The current 5-day TP lifespan recommendation is based on factors V, VII, VIII activity levels at outdate,⁵ however, the multitude of plasma proteins participating in attenuating fibrinolysis and correction of TIC remain unknown, as does their change in activity level over the thawed storage time.

Plasma has been a well-known buffer of the circulatory system since the 1950s⁸ and more recently has been appreciated to also buffer the systemic fibrinolytic activity.³ The adverse effects of hemodilution of this delicate system in trauma patients with crystalloid and colloid fluids are well documented. *In vitro* albumin and artificial colloid hemodilution decrease the rate of clot initiation and formation as well as maximum clot strength.^{9,10} Previous studies demonstrated an increased risk of hyperfibrinolysis in trauma patients with each liter of prehospital crystalloid fluid,² and *in vitro* studies have confirmed this association.^{3,11}

Tissue-plasminogen activator (tPA) is a serine protease known to catalyze the reaction of plasminogen to plasmin leading to the breakdown of blood clots. High tPA activity levels drive hyperfibrinolysis in trauma patients.^{12,13} Our group has previously described an *in vitro* assay adding tPA to whole blood to assess the impact of clot fibrinolysis with thrombelastography (TEG) for the evaluation of different resuscitation fluids.³ Using this assay, we hypothesize that TP retains the ability to enhance clotting and correct TIC and tPA-mediated fibrinolysis at 14 days storage at 1–4°C similar to FFP and superior to albumin and normal saline.

Materials and Methods

Materials

Human single-chain tissue plasminogen activator (tPA) from Molecular Innovations (Novi, MI) was diluted with 5% bovine serum albumin in phosphate-buffered solution (PBS) followed by separation into aliquots and storage at -80°C . Pooled AB blood type FFP was a generous gift from Terumo Blood Component Technology (Lakewood, CO). Normal saline was purchased from Baxter International (Deerfield, IL). 5% Human Albumin was purchased from Grifols Biologicals Inc. (Los Angeles, CA) and stored at $1-4^{\circ}\text{C}$ until use.

Methods

Healthy volunteer blood collection—After informed consent was obtained, blood samples were collected from eight healthy volunteers in 3.3-ml buffered sodium citrate (3.2%) tubes (Vacutainer, Becton-Dickinson, Franklin Lakes, NJ) under the Colorado Multiple Institutional Review Board (COMIRB) protocol number 10-0477. Volunteers were 75% men, ages 25–42, not smokers, not obese, not pregnant, or taking any medications at the time of blood draw ($n=8$).

Plasma—One unit of pooled AB fresh frozen plasma (FFP) from ten donors was thawed and separated into 500ul aliquots followed by flash freeze with liquid nitrogen. Samples were subsequently thawed in a 37°C water bath and stored at $1-4^{\circ}\text{C}$ at the following intervals: 14, 10, 7, 5, 3, and 1-day (TP) prior to the experiment.

Thrombelastography—To simulate a prolonged transport time and large volume resuscitation, blood samples underwent a 50% dilution with the above TP intervals as well as FFP, normal saline (NS), albumin, and whole blood (WB) control. Citrated native and a tPA-challenge (75ng/ml) TEG were run for each dilution as previously described using the TEG 5000 Thrombelastograph Hemostasis Analyzer (Haemonetics, Niles, IL).³ TEG properties—speed of clot initiation (R-time), rate of clot formation (angle), maximum clot strength (maximum amplitude, MA), and percent lysis at 30 minutes (LY30) were analyzed.

Statistics—Sample size was calculated using PASS14 (NCSS, LLC) based on an adequate power (0.9) for a non-inferiority paired study for the main outcome LY30. Based on previous analysis of 160 healthy volunteer tPA-challenge TEGs (75ng/ml), the median LY30 was 8.3% with a standard deviation of 9.9% and 95th percentile of 27% (at which point treatment would be initiated for hyperfibrinolysis). A sample size of 8 pairs allowed for an equivalence test with limits -2.6 to 2.6 (5.2%) with 90% power using a 5% significance level.

Values are reported as median with interquartile ranges. Statistical analysis was done using SPSS version 24 (IBM). TEG values R-time, angle, MA, and LY30 had a skewed, non-normal distribution. Differences across dilution groups were detected using a non-parametric paired Friedman test. A Bonferroni correction of 4 was applied (for comparison of 14-day TP to FFP, WB, albumin, and NS) and a statistically significant p value was set at 0.0125. Post-hoc analysis was done using a Wilcoxon signed ranks test. To determine whether a

correlation between storage time and clot properties existed, a Spearman correlation was done for R-time, angle, MA, and LY30.

Results

Citrated native TEG properties: resuscitation fluids impact clot formation, not degradation

Without the addition of tPA, time to clot initiation (R-time), speed of clot formation (angle), and maximum clot strength (MA) were all significantly different among the different resuscitation fluids ($p < 0.0002$ for all). 14-day TP and FFP stimulated significantly shorter time to clot initiation compared to WB, NS, and albumin ($p = 0.012$ for all comparisons, Table 1). R-time of 14-day TP was similar to FFP (Table 1). 14-day TP and FFP also stimulated faster clot formation (angle) compared to WB, NS, and albumin ($p = 0.012$ for all comparisons). 14-day TP and FFP exhibited a stronger maximum clot strength (MA) compared to WB, NS, and albumin ($p = 0.012$ for all, Table 1). In the absence of tPA, clot breakdown (LY30) did not demonstrate a significant difference across dilution groups ($p = 0.035$, Table 1).

tPA-challenge TEG properties differ across dilution groups, but not within TP groups

In the presence of tPA, resuscitation fluids significantly impacted all TEG properties analyzed (Table 2, Figure 1). R-time significantly differed across dilution groups ($p = 0.0004$) with, again, shorter times to clot initiation with FFP and 14-day TP compared to WB, NS, and albumin ($p = 0.012$ for all comparisons). Similar to citrated native, angle and MA were significantly different across dilution groups ($p = 0.0002$, $p < 0.0001$, respectively). FFP and 14-day TP demonstrated faster clot formation (angle) compared to WB, NS, and albumin ($p = 0.012$ for all). FFP and 14-day TP also had increased maximum clot strength compared to WB, NS, and albumin ($p = 0.012$ for all). Lastly, clot breakdown (fibrinolysis, LY30%) was significantly different across dilution groups in the presence of tPA ($p = 0.001$) with FFP and 14-day TP demonstrating significantly less clot breakdown compared to NS and albumin ($p = 0.012$ for all) (Figure 1). Figure 2 demonstrates a sample tPA-challenge TEG tracing.

Thawed plasma clotting properties retained over 14 days

The power of this experiment was designed to detect clinically significant differences in the clot breakdown of tPA-challenge TEG (LY30). In the presence of tPA, 14-day thawed plasma did not demonstrate a statistically significant difference in clot breakdown (LY30) as compared to FFP ($p = 0.017$) or WB ($p = 0.05$). FFP trended toward reduced clot breakdown compared to 14-day TP (median 5.6% and 9.6%, respectively), while 14-day TP trended toward decreased fibrinolysis (LY30) compared to WB (14.6%, $p = 0.05$). Clot properties across dilution groups were similar between thawed plasma dilutions and FFP (Table 2, Figure 3). There was no significant correlation between storage time and clot properties including R-time ($p = 0.633$), angle ($p = 0.967$), MA ($p = 0.179$), and LY30 ($p = 0.320$) in the presence of tPA (Figure 3).

Discussion

Replacement of whole blood by 50% with plasma stored up to 14-days attenuates tPA mediated clot degradation when measured by thrombelastography. This 14-day old thawed plasma was also associated with a shorter clot initiation than whole blood, a faster clot generation, and an overall increase in clot strength. This *in vitro* data supports increasing the lifespan of TP in the treatment of TIC, without the concerns of a significant loss of hemostatic properties. This would lead to greater availability of TP at smaller hospitals and limit the amount of plasma wastage at larger hospitals with TP currently available.^{14,15}

Plasma replacement early after injury has been associated with an improvement in survival,^{16,17} however, the mechanism for this remains uncertain. Historically, 30% of normal clotting factor activity has been considered sufficient for hemostasis.¹⁸ Previous work has shown a 35–48% decrease in Factor V and 45–52% decrease in Factor VIII over 2-week storage of thawed plasma.^{5,19} Matijevic *et al* noted a 20–30% decrease in these factor activity levels at 5-days resulted in 40% increase in R-time and a 60% decrease in thrombin generation by thrombin generation assay.²⁰ Discrepancy between our two studies correlates with the complexity of the coagulation system in the injured patient, with endotheliopathy, coagulation factor depletion, hypothermia, hypotension, tissue injury, and the procoagulant/anticoagulant balance all playing a role. In our hands, a 50% dilution of whole blood with normal saline or albumin did not result in prolongation of the R-time. Prior TIC studies have failed to identify a critical depletion of coagulation factors as the sole cause of trauma-induced hypocoagulability.^{21,22}

The transition of large trauma centers to thawed plasma has led to a near 50% decrease in time to plasma transfusion^{23,24} resulting in an independent predictor of decreased mortality.²³ The trade-off with the increasing use of thawed plasma is the 2.2% wastage rate, most of which is limited AB donor plasma. The AB blood type accounts for only 3% of the population but up to 25% of all plasma transfused, with higher proportions in trauma patients.²⁵ Initial implementation of the increased thawed plasma lifespan (from 24 to 96 hours) significantly decreased discarded plasma,^{14,15} and a similar decrease in discard rate of this valuable resource could be expected with expansion to 14 days. And while certain coagulation factors are known to lose significant activity over a 14-day thawed storage time,^{5,19} we have shown that a substantial dilution with 14-day TP resulted in enhancement of clot formation compared to whole blood.

In our current study, we were unable to identify consistent trends in clotting properties over the 14-day storage of thawed plasma to correspond to the presumed decrease in clotting factor activity (Figure 3). While there are slight differences among the time to clot initiation (R-time) and rate of clot formation (angle) over the duration of storage (Figure 3, Table 1, 2), these values all remain hypercoagulable compared to whole blood (shortened R-time, increased angle) without a consistent trend. Possible reasons for the lack of trend in clotting function over thawed storage are the minimum coagulation factor activity levels are actually lower than what is currently thought, and so the addition of even 14-day thawed plasma results in excess factor activity to form a functional clot. Another possibility is there are

additional proteins responsible for clot formation and stabilization in plasma that do not actually degrade quickly during thawed storage.

We acknowledge limitations to this study. While increased tPA activity has been repeatedly shown to be present in the plasma of trauma patients,^{12,13} there are undoubtedly other clotting abnormalities that may impact the fibrinolysis profile. Our addition of *in vitro* exogenous tPA to whole blood dilutions does not completely replicate the plasma changes of trauma patients, but does produce a reliable fibrinolysis profile to study the effects of interventions. Second, the pooled donor plasma was thawed, aliquoted, and refrozen followed by a second thawing for storage and use. A second freeze thaw cycle likely further decreased protein activity level, although we showed retention of plasma clotting properties despite a second thaw cycle. Another limitation is the *in vitro* nature of the study. Further confirmatory *in vivo* experiments are needed prior to increasing the duration of thawed plasma.

Conclusion

We have shown preservation of clot formation and inhibition of clot degradation over a 14-day storage of TP by TEG similar to FFP and superior to normal saline and albumin. While further studies are needed to confirm the applicability, consideration should be made to increase the duration of stored TP as this will decrease the outdated wastage rate as well as increase the availability of TP at smaller hospitals.

Acknowledgments

There are no acknowledgements for this manuscript.

Research reported in this publication was supported by the National Institute Of General Medical Sciences of the National Institutes of Health under Award Number T32 GM008315 and P50 GM049222. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Additional research support was provided by Haemonetics Corporation (Niles, IL). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or Haemonetics.

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Figure 1.

Box-plot tPA-challenge TEG properties of thawed plasma (TP), whole blood (WB), normal saline (NS), and albumin. Plasma dilution (FFP, TP) resulted in reduced R-time, increased angle, and increased MA compared to WB, NS, and albumin. Plasma as well as whole blood resulted in significantly reduced clot breakdown (LY30%) compared to NS, albumin.

*p=0.012 compared to WB, NS, albumin. †p=0.012 compared to NS, albumin.

Figure 2. Sample tPA-challenge TEG tracing for whole blood (brown), FFP (black), 14-day TP (blue), albumin (pink), and normal saline (NS, green). NS and albumin demonstrate decreased angle, MA, and increased LY30.

Figure 3.

Storage time does not lead to clot property changes. Storage time does not correlate with R-time (0.077, $p=0.633$), angle (-0.007 , $p=0.967$), MA (0.214, $p=0.179$), or LY30 (0.159, $p=0.320$) in the presence of tPA. Values reported as median $\pm$ SD.

Table 1
Citrated native TEG properties

Dilution of whole blood (WB) with thawed plasma (TP), normal saline (NS), and albumin resulted in distinct TEG tracings. FFP and all of the TP intervals demonstrated similar TEG properties, with similar R-time, angle, MA, and LY30. TP and FFP have shorter time to clot initiation (R-time), faster rate of clot formation (angle), and stronger maximum clot strength (MA) compared to WB, NS, and albumin. Values reported as median (interquartile range).

	R-time (min)	Angle (degrees)	MA (mm)	LY30 %
Whole blood	11.8 (11.4–15.4)	36.1 (29.6–45.8)	50.0 (48.0–52.3)	0.7 (0.2–1.6)
Normal Saline	11.2 (10.6–13.3)	42.4 (40.4–50.2)	42.9 (39.4–48.5)	0.1 (0–3.3)
Albumin	11.8 (10.5–13.5)	35.6 (33.5–44.1)	40.5 (38.8–43.9)	0.4 (0.1–5.4)
FFP	9.1 (7.3–10.2) *	64.6 (62.3–67.4) †	60.9 (59.6–64.2) ‡	1.5 (0.3–2.1)
1-day TP	8.6 (7.6–8.8) *	66.6 (58.9–68.7) †	61.4 (61.0–62.5) ‡	1.8 (1.1–2.6)
3-day TP	8.8 (8.4–9.5) *	67.1 (61.1–69.9) †	65.5 (63.8–67.5) ‡	1.7 (1.0–2.8)
5-day TP	8.5 (8.2–8.8) *	66.1 (60.9–69.8) †	63.0 (60.8–64.0) ‡	1.7 (1.4–2.8)
7-day TP	10.0 (6.9–10.2) *	62.7 (61.3–69.7) †	63.5 (61.0–65.0) ‡	1.7 (1.0–2.8)
10-day TP	7.6 (6.4–9.8) *	63.8 (62.4–69.1) †	62.0 (59.3–62.8) ‡	1.6 (1.1–4.4)
14-day TP	10.0 (9.2–11.2) *	62.7 (54.0–66.1) †	62.5 (60.0–64.0) ‡	1.7 (1.5–3.3)

* p=0.012 compared to WB, NS, albumin R-time.

† p=0.012 compared to WB, NS, albumin angle.

‡ p=0.012 compared to WB, NS, albumin MA.

Table 2
tPA-challenge TEG properties of whole blood, dilution groups

In the presence of tPA, FFP and TP dilutions had shorter time to clot initiation (R-time) and faster speed of clot formation (angle) as well as stronger maximum clot strength (MA). NS and albumin dilution demonstrated significant clot breakdown in the presence of tPA (LY30%) while TP and FFP dilutions inhibited this clot breakdown. Values reported as median (interquartile range).

	R-time (min)	Angle (degrees)	MA (mm)	LY30%
Whole blood	9.8 (8.9–13.4)	43.6 (37.3–51.3)	50.2 (46.8–51.6)	14.6 (4.7–17.9) ^{\$}
Normal saline	8.7 (8.5–10.8)	41.6 (35.7–46.7)	32.3 (23.1–35.4)	58.1 (45.0– 64.3)
Albumin	10.1 (8.5–12.4)	34.8 (33.6–42.7)	21.6 (18.9–35.8)	59.3 (49.3–72.5)
FFP	7.5 (6.3–7.8) *	67.0 (64.7–69.4) †	60.9 (58.3–63.3) ‡	5.6 (4.5–8.6) ^{\$}
1-day TP	7.2 (6.6–7.3) *	68.9 (65.1–70.2) †	60.0 (58.0–62.0) ‡	8.8 (7.4–12.4) ^{\$}
3-day TP	7.5 (6.9–8.0) *	66.1 (61.8–69.4) †	61.0 (58.8–63.3) ‡	9.4 (7.7–11.8) ^{\$}
5-day TP	6.5 (6.4–7.1) *	68.1 (67.4–70.1) †	62.5 (60.3–64.0) ‡	10.5 (6.9–11.1) ^{\$}
7-day TP	7.1 (6.0–9.5) *	65.6 (62.6–70.2) †	62.5 (60.0–62.8) ‡	6.7 (3.5–8.1) ^{\$}
10-day TP	6.2 (5.2–7.8) *	67.1 (65.9–72.3) †	61.5 (60.3–64.5) ‡	7.4 (6.4–8.8) ^{\$}
14-day TP	7.2 (6.9–7.8) *	67.1 (63.9–67.7) †	62.5 (59.0–63.0) ‡	9.6 (8.0–10.4) ^{\$}

* p=0.012 compared to WB, NS, albumin R-time.

† p=0.012 compared to WB, NS, albumin angle.

‡ p=0.012 compared to WB, NS, albumin MA.

^{\$} p=0.012 compared to NS, albumin LY30.