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In Vitro Effects of Thawing Fresh-Frozen Plasma at Various Temperatures

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Summary: Thawing fresh-frozen plasma (FFP) in South Africa is uncontrolled because the plasma is issued frozen from the blood bank and thawing techniques and temperatures are at the discretion of the clinician. Following anecdotal reports of disseminated intravascular coagulation (DIC) developing in patients who received FFP thawed in an uncontrolled manner, the effects of various thawing temperatures on coagulation parameters were studied. Fifteen adult units of FFP were each divided into 4 satellite units by the South African Blood Transfusion Service before freezing at –25°C. These bags were then defrosted in a waterbath at 22°C, 37°C, 45°C and 60°C, respectively, and removed as soon as they had thawed. Samples were collected for measurement of International Normalized Ratio (INR), prothrombin time (PT), partial thromboplastin time (PTT), fibrinogen, and D-dimers. These tests were done according to standard operating procedures. FFP samples were also used in place of agonist in platelet aggregation studies to assess whether the FFP could induce platelet aggregation. Results were analyzed with the percentage similarity model. Using this method the percentage similarity (%sim) of each bag thawed at each temperature with the same donor's bag thawed at 37°C was calculated. The mean, standard deviation, and percentage coefficient of variation of the percentage similarities were then derived. Data sets were also compared using the Wilcoxon test. The fibrinogen values remained stable at 22–45°C (%sim = 97–99%) while there was a significant decrease in fibrinogen levels at 60°C compared with 37°C ($p<0.001$, %sim = 75%). INR and PTT values were highest in the bags thawed at 60°C (%sim = 114% and 110%, respectively) with the difference between the INR levels at 60°C compared with 37°C showing statistical significance ($p<0.05$). D-dimers were high at all temperatures tested with widely ranging results at each temperature. The FFP did not induce platelet aggregation at any of the thawing temperatures. In summary, INR and PTT values increase at a thawing temperature of 60°C compared with 37°C. D-dimers are elevated in thawed FFP. Fibrinogen levels are markedly decreased in FFP thawed at 60°C compared with that thawed at 37°C. FFP should be thawed at 37°C in a strictly controlled environment.
Key Words: Fresh frozen plasma—FFP—Thawing—Temperature—DIC—Thaw.

Thawing of fresh-frozen plasma (FFP) in South Africa is uncontrolled because plasma is issued frozen by the blood bank and thawing techniques and temperatures are at the discretion of the clinician. We have received anecdotal reports of disseminated intravascular coagulation (DIC) developing in patients who received FFP that had been thawed in an uncontrolled manner in hot water. It is conceivable that exposure of the coagulation factors in FFP to high temperatures might either denature the proteins, reducing the efficacy of the FFP, or activate the proteins, precipitating activation of the coagulation cascade and potentially causing DIC in the patient.

Under physiologic conditions, activation of the coagulation cascade leads to the conversion of prothrombin to thrombin, which catalyzes the conversion of fibrinogen to fibrin. Fibrin binds to platelets and stabilizes an initial platelet clot.

The opposing physiologic process is fibrinolysis, during which the clot is progressively dissolved by plasmin, resulting in the formation of fibrin breakdown products known as D-dimers.

In pathologic conditions, such as DIC, there is continuous activation of the clotting cascade

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with resultant ongoing fibrinolysis. Biologic consequences of DIC are fibrinogen deficiency and overconsumption of factors that may result clinically in a diffuse hemorrhagic syndrome. FFP is infused to patients who have a deficit in coagulation factors and/or fibrinogen, as occurs during DIC, to replace the missing proteins. It is thus crucial to maintain high levels of fibrinogen and coagulation factors in FFP. A low level of these proteins (such as that caused by previous denaturation or consumption) renders FFP unsuitable for therapeutic administration.

A commonly used assay for the consumption of coagulation factors is to measure coagulation parameters such as partial thromboplastin time (PTT), prothrombin time (PT), and International Normalized Ratio (INR). Consumption of the key factors of coagulation will result in prolongation of one or more of these assays.

Previous studies have found no significant differences in coagulation parameters (PT, PTT, fibrinogen levels, and factor levels) between bags of FFP thawed at 37°C compared with those thawed at 45°C or 56°C (1,2). We chose to reassess these parameters by performing assays on satellite bags of FFP from the same donor at thawing temperatures of 20°C, 37°C, 45°C, and 60°C.

The formation of cross-linked fibrin during a coagulation process can be detected by the consumption of fibrinogen as well as by the release of products of fibrin degradation, namely D-dimers. Both these variables were measured in this experiment as lower levels of fibrinogen after thawing of the FFP might have meant only that fibrinogen had been denatured. This variable alone could not be used to determine whether cross-linked fibrin had in fact been formed during the thawing process. D-dimers are a specific test for the formation of cross-linked fibrin. It was thus postulated that if the fibrinogen decreased during thawing in conjunction with an elevation of D-dimers, one could conclude that there had been activation of the coagulation cascade during the thawing process.

The level of thrombin, one of the end products of coagulation, cannot be assayed easily using our routine laboratory techniques. We therefore used indirect methods to look for the presence of activated thrombin. Platelets are known to aggregate in the presence of different activators, the most potent being collagen and thrombin. We hypothesized that if the thawed FFP contained activated factors, it would also contain activated factor II, thrombin, and therefore could trigger platelet aggregation. We thus used the thawed FFP in place of an agonist in platelet aggregation studies to assess whether the FFP could precipitate platelet aggregation.

MATERIALS AND METHODS

Preparation of FFP

FFP was prepared by the South African Blood Transfusion Services according to their standard operating procedures. Blood units were centrifuged using either a Hettich or Sorval blood bag centrifuge and plasma was separated from whole blood using the Optipress system. A quad pack of PL1240 bags were connected to the plasma unit using a Terumo Sterile Connecting Device. The contents of the plasma unit (220–290 mL) were separated equally into the attached 4 bags. The aliquoted units were labelled and detached from each other with a heat sealer and the units were frozen using the blast freezing method. The units were stored below –25°C.

Thawing of FFP

FFP was thawed in a thermostat-controlled waterbath (Gesellschaft Fur Labortechnik, Oberschleibheim, Germany) and water temperature was confirmed with an additional thermometer (Lloyd Register Quality Co., Coventry, UK). Thawing times were recorded but are not reported as they are expected to vary depending on the volume in each bag. There was no manual agitation of the bags except the minimum required to determine whether the contents were fully thawed. Bags were removed as soon as they were judged fully thawed.

Measurement of INR, PTT, D-dimers, and Fibrinogen

INR, PTT, D-dimers, and fibrinogen levels were measured according to the standard operating procedures of the Johannesburg Hospital Haematology Laboratory. INR, PTT, and D-dimers were tested using the ACL Futura (Ilex Medical Systems, Milan, Italy). Fibrinogen assays were run on the ACL 7000 (Ilex Medical Systems, Milan, Italy). If the fibrinogen level of a particular sample was not detectable, that sample was diluted 1:2 with standard plasma of known fibrinogen level. If the fibrinogen level of the diluted sample was less than or equal to half of the fibrinogen level in the undiluted standard plas-

ma, the fibrinogen level of the original sample was taken to be zero. This was recorded as 0.1 g/L for statistical purposes.

Platelet Aggregation Studies

A Chronolog whole blood aggregometer (Beckman Coulter, Haventown, PA) was used for platelet aggregation studies. Blood samples from normal controls (volunteers) were collected into citrated tubes to make platelet-poor plasma (PPP) and platelet-rich plasma (PRP). To attain PPP, the blood was centrifuged at 2060 g for 10 minutes in a GS-6R centrifuge (Beckman, Miami, FL) with brakes on. Platelet counts were measured and accepted if $<10 \times 10^9$/L. Blood was centrifuged at 130 g for 30 minutes with the brakes off to make PRP. Platelet counts were accepted if they were 180–260 $\times 10^9$/L. To ensure the reliability of aggregation studies, PRP was discarded after 3 hours of use and new PRP was constituted. A control aggregation was performed on each new batch of PRP using collagen (final concentration 2 μg/mL). For each assay, 50 μL of thawed FFP was used with 450 μL of PRP.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel 2000 (Microsoft, Seattle, Washington). The mean PT, PTT, INR, D-dimers, and fibrinogen values were calculated for each temperature and standard deviations were derived. We also compared each bag with the satellite bags from the same donor using the percentage similarity model (3). For each bag at each temperature, the percentage similarity of the test value was compared with the value of that donor's bag at 37°C. This is done by taking an average of the 2 values, dividing it by the value at 37°C and expressing this quotient as a percentage $(((0.5(V_{37} + V_t))/V_{37}) \times 100$ where V_t indicates test value) The mean, standard deviation, and coefficient of variation were then calculated for the percentage similarities at each temperature.

Further statistical analysis included performance of the Wilcoxon paired non-parametric test using GraphPad Prism (V4.00 for Windows, GraphPad Software, San Diego, California).

RESULTS

INR and PTT Results

INR and PTT tests were performed on 15 donors' bags. Two bags were excluded because they failed to clot at a particular temperature. This failure was at 45°C and 60°C, respectively. None of the other 14 bags failed to clot at these temperatures. These outlying results were thus deemed to be due to laboratory error.

The mean percentage (%) similarities of the 22°C and 45°C groups showed that the INR and PTT values of these groups were close to that of the 37°C group (99% to 104% similar) (Tables 1 and 3). The %CV of the 45°C INR group was 10.1, indicating a wide spread of %similarity results, despite a mean %similarity of 100%. AT 60°C, the %similarities were 114% and 110% in the INR and PTT groups, respectively, indicating higher values on average than those found in the 37°C group (%CV 19.8 and 15.4, respectively).

Using the Wilcoxon paired non-parametric test, the median INR value at 60°C was found to be significantly different from that at 37°C

TABLE 1. INR and PTT Results (n=13) of FFP Thawed at 60°C, 45°C, 37°C, and 22°C

Parameter	60°C	45°C	37°C	22°C
INR				
Mean	1.38 (0.88–2.78)	1.09 (0.95–1.56)	1.09 (0.88–1.28)	1.17 (0.86–1.59)
SD	0.48	0.17	0.11	0.17
PTT (secs)				
Mean	39.38 (27.3–48.4)	33.06 (23.5–41.5)	33.11 (23.3–43.7)	32.58 (22.7–44.0)
SD	10.15	5.04	5.82	5.69

INR, international normalized ratio; PTT, prothrombin time; SD, standard deviation; n, sample number.

($p<0.03$). The median PTT value at 60°C did not show significant difference from that at 37°C. There was no significant difference between the INR or PTT values of the 45°C group when compared with the 37°C group. There were significant differences ($p<0.05$) noted between median INR values of the 22°C group when compared with the 37°C group or the 45°C group as well as between the 45°C and 60°C groups. Significant differences ($p<0.05$) in PTT values were noted when comparing the 22°C group with the 37°C group, as well as between the 60°C group and the 22°C or 45°C groups.

Fibrinogen Results

Fibrinogen results were calculated for 15 donors' bags. The highest mean fibrinogen values were seen at 37°C (3.98 g/L) and 45°C (3.90 g/L; mean %sim=99, %CV=2.4) (Tables 2 and 3). The lowest mean value was observed in the 60°C group (1.9 g/L; mean %sim75, %CV=22.8). At 60°C, 4 bags had no measurable fibrinogen.

Using the Wilcoxon test, the median fibrinogen value in the 60°C group was found to be significantly different to the value at any of the other three temperatures ($p<0.001$). The median fibrinogen value in the 22°C group was also significantly different ($p<0.001$) from that of any of the other 3 groups. There was no significant difference ($p<0.05$) found between the 45°C and 60°C groups.

D-dimer Results

Five donor bags were excluded from D-dimer measurement. These were from a group of 45°C bags assayed on the same day and all gave an "error" reading. The control PPP from a healthy donor (never heated) also gave an "error" reading. None of the bags from these 5 donors gave D-dimer "error" readings at any other temperature. None of the bags from the other 10 donors gave "error" readings at 45°C. This group of readings was thus considered to be due to laboratory error.

D-dimer results were thus calculated from ten donor bags. At 22°C and 37°C, all these D-dimer values were <1.0 mg/L except for 1 bag. That bag had high readings at 22°C and 37°C (6.360 and 5.895 mg/L, respectively) but low readings (<1.0 mg/L) at 45°C and 60°C. At 45°C and 60°C, all bags had values <1 mg/L except for 1 bag. That bag had values of 6.64 mg/L and 6.56 mg/L at 45°C and 60°C, respectively but normal D-dimers at 22°C and 37°C. These outlying values were not excluded but considered in the calculations. We did not believe that they were due to laboratory error but rather due to heat, manipulation, or unknown variables causing activation of the plasma.

The lowest mean D dimers were seen in the 37°C group (0.789 mg/L). The 22°C group had a mean D-dimer value of 0.911 mg/L (mean %sim=119, %CV=19), the 60°C group had a mean value of 0.887 (mean %sim=355, %CV=229.6) and the 45°C group had the highest mean value of 0.916 mg/L (mean %sim=374, %CV=821). The extremely high %CVs reflect the large spread of results caused by the 1 outlying value at each temperature. Using the Wilcoxon test the only groups between which a significant difference was found were the 22°C and 37°C group ($p<0.005$).

Platelet Aggregations

Platelet aggregations using FFP as an agonist were performed for 10 donors' bags at 37°C, 45°C, and 60°C. The FFP did not stimulate platelet aggregation at any of these temperatures.

TABLE 2. Fibrinogen (n=15) and D-Dimer (n=10) Results of FFP Thawed at 60°C, 45°C, 37°C, and 22°C

Parameter	60°C	45°C	37°C	22°C
Fibrinogen (g/L)				
Mean	1.9 (0.10–3.39)	3.9 (2.74–5.71)	3.98 (2.76–6.05)	3.73 (2.55–5.52)
SD	1.3	1.01	1.07	1.02
D-dimers (mg/L)				
Mean	0.887 (0.091–6.558)	0.916 (0.138–6.641)	0.789 (0.090–5.895)	0.911 (0.113–6.36)
SD	2.01	2.02	1.81	1.93

SD, standard deviation; n, sample number.

DISCUSSION

Mean INR, PTT, and fibrinogen results were within physiologic reference ranges when the FFP was thawed between 22°C and 45°C. The INR and PTT were prolonged in the 60°C group with mean values marginally outside of the laboratory's normal range (normal range INR: 0.94–1.30; PTT 23.0–39.0 seconds). This may be due to either denaturation of the clotting factors or due to their consumption by activation of the clotting cascade. Mean fibrinogen values were markedly decreased in the 60°C group with 4 of 15 bags containing no measurable fibrinogen at all. This may again be due to denaturation or consumption of the fibrinogen. This is therefore a strong indication that 60°C is an unacceptable temperature at which to thaw FFP (Table 2).

D-dimers were elevated above the laboratory's reference range (0.0–0.2 mg/L negative, 0.2–0.4 mg/L indeterminate) in the samples from all temperatures studied; however, the lowest mean D-dimers were recorded in the 37°C group. This suggests that fibrin clot formation and breakdown occurs during thawing of FFP at any temperature, but is more prominent at temperatures differing from body temperature. The statistical analysis should, however, be interpreted with caution due to the wide range of results recorded at each temperature (including 37°C) and the outlying values at each temperature that were included in the analysis.

We were not able to demonstrate that FFP thawed at any temperature studied could cause platelet aggregation. Although an explanation would be that the clotting factors were rather denatured and not activated by the heating process, this does not explain the increased D-dimers. It may be that a higher concentration of FFP added to the PRP is required to cause aggregation. Fifty microliters FFP with 450 μL PRP was chosen simply because higher concentrations of FFP caused the initial absorbance change to move the needle of the recorder off our graph paper. Settings could have been adjusted to allow higher concentrations. The most plausible explanation is that any clotting factors activated by the heating process were consumed in the coagulation cascade during heating, leaving none remaining to trigger platelet activation. This is substantiated

TABLE 3. % Similarity Results of FFP Thawed at 60°C, 45°C, and 22°C using 37°C as a Standard

Parameter	60°C	45°C	22°C
INR			
Mean	114	100	104
SD	23	10	4
%CV	19.8	10.1	4
PTT			
Mean	110	100	99
SD	17	6	3
%CV	15.4	5.9	2.7
Fibrinogen			
Mean	75	99	97
SD	17	2	2
%CV	22.8	2.4	2.4
D-dimers			
Mean	355	374	119
SD	815	821	19
%CV	229.6	219.8	15.9

INR, international normalized ratio; PTT, partial thromboplastin time; SD, standard deviation; %CV, coefficient of variation.

by the presence of D-dimers indicating previous clot formation and breakdown. This would however have had to happen on a very small scale if at all, as there were no macroscopically visible fibrin strands produced in the FFP and a critical concentration high enough to cause consumption of all the clotting factors was not reached (as demonstrated by INR and PTT assays which did not fail to clot).

In the experiment, the FFP bags were carefully monitored during thawing and removed as soon as they were judged completely thawed. This is unlikely to happen in a clinical setting where bags may be left in water to thaw for some time after complete defrosting. This would be likely to affect results in bags thawed at high temperatures. While the differences between the 45°C and 37°C groups were slight in this experiment and did not reach statistical significance using the Wilcoxon method, it was noticed in preliminary experimentation that bags left longer in hot water became more opaque and contained more sediment. This might be caused by complete denaturation of fibrinogen and clotting factors and may cause dramatic changes in D-dimer levels. We hesitate to recommend 45°C as a suitable thawing temperature for this reason. This should be explored further in studies that leave FFP at 45°C for a prolonged time after thawing.

CONCLUSION

A high thawing temperature (60°C) caused prolongation of the mean INR and PTT values of the FFP. It decreased the fibrinogen level (to zero in some cases) and increased the D-dimer level. An exaggeration of these effects is expected if the FFP is allowed to remain at the warm temperatures after it has fully defrosted. Optimal values were obtained at the most physiologic temperature tested, namely 37°C. We therefore recommend that thawing of FFP should be done in a 37°C waterbath in a strictly controlled environment. Further studies evaluating 45°C as an acceptable temperature should be conducted.

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