Vitamin K-dependent coagulation factors and fibrinogen levels in FFP remain stable upon repeated freezing and thawing

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BACKGROUND: FFP is considered adequate for transfusion up to 24 hours after thawing and is currently used most often to replace deficient clotting factors, such as in warfarin overdose. We set to examine the levels of vitamin K-dependent factors (i.e., prothrombin, FVII, F IX, FX), as well as fibrinogen, upon twice freezing and thawing of FFP. If factor levels in refrozen FFP remain within normal limits, this component can possibly be transfused, thus avoiding wastage of precious blood components.

STUDY DESIGN AND METHODS: Twenty units of FFP, five units of each blood group A, B, AB, and O, were thawed, and aliquots were taken for measurement of coagulation factors. The plasma units were then kept for 24 hours at 4°C, at which point a second aliquot was taken, The remaining FFP units were refrozen and kept at –80°C for 1 week. The above procedure was then repeated. Coagulation-factor activity and fibrinogen level were measured by the coagulation analyzer.

RESULTS: The mean levels of prothrombin, FVII, F IX, FX, and fibrinogen of each blood group (A, B, AB, and O) were calculated for each of four time points and found not statistically different ($p > 0.05$). Therefore, the rest of the analysis was done for all 20 FFP units as one group. The mean ± SD levels of each coagulation factor at each time point demonstrated that all levels were within normal limits of all factors measured and that for none of the factors was there a significant decay of activity.

CONCLUSIONS: The levels of prothrombin, FVII, F IX, FX, and fibrinogen remain stable and adequate for transfusion in twice-thawed-and-refrozen FFP. This component can be safely used for transfusion as a source of vitamin K-dependent clotting factors and fibrinogen.

FFP is considered adequate for transfusion immediately after thawing or for 24 hours if kept at 1 to 6°C.[1] Current indications for transfusion of FFP as hemostatic support include replacement of deficient coagulation factors,[1–3] as in warfarin treatment,[4] liver disease[5] massive transfusion, and consumption coagulopathy.[6]

It has been shown that FVIII loses activity if separation and freezing of plasma are not performed within 6[7] to 8[8] hours of blood collection, while the levels of FV, FVII, and FXI,[7] as well as FX, fibrinogen, AT III, protein C, and protein S,[8] remain stable in whole blood at 1 to 6°C for 24 hours.

In AABB Standards, a component called "thawed plasma" exists. As opposed to FFP, which is thawed less than 24 hours before transfusion, this component has been kept thawed at 1 to 6°C for up to 5 days.[9]

Indeed, it has recently been shown[10] that FII, FV, FVII, FX, and fibrinogen are stable when kept at 4°C for at least 3, and possibly 5, days, while FVIII loses activity.

As opposed to clotting factors losing activity in refrigerator conditions, cold activation of coagulation zymogens in transfusion components is unwarranted, posing a hazard for thrombosis and disseminated intravascular coagulation (DIC). FVII was known to become activated in plasma under refrigerator conditions. Seligsohn et al.[11] looked at FVII activation in tubes versus blood-bank bags and found that FVII activation was negligible in blood bank conditions, making storage of FFP for 24 hours in the cold safe for transfusion.

The stability of coagulation proteins upon twice freezing and thawing FFP was examined by Dzik et al.,[12] who showed that the levels of FV and FVIII decrease sig-

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nificantly, with a slight but statistically valid prolongation of the prothrombin time (PT) and activated partial thromboplastin time (aPTT) and a decrease in the FV and FVIII:C levels compared with control plasma, but concluded that measured deterioration in coagulation of twice-frozen FFP is unlikely to be of clinical importance.[12]

However, under routine blood-bank work and standard operating procedures, if FFP has been thawed and not transfused within 24 hours, it is usually discarded. In large medical centers with extensive surgical and trauma activity, this creates a meaningful waste of resources.

Therefore, we set to examine the fate of vitamin K-dependent factors (namely prothrombin, FVII, F IX, FX) and fibrinogen upon twice freezing and thawing and keeping FFP for 24 hours at 4°C, ready for use, between the two freezing procedures.

MATERIALS AND METHODS

Preparation and storage of FFP

Twenty FFP units, five of each blood group (A, B, AB, O), were purchased from MADA (Magen David Adom, the Israel central blood collection organization). Whole blood was collected in CPDA-1 triple bags (Teva Medical LTD, Ashdod, Israel) from normal donors and maintained at 18°C for 18 to 24 hours on cooling trays (Compocool, Fresernius/NPBI, Essen Compasam, Holland). Separation of plasma was performed by two centrifugation steps, according to AABB.[1] FFP units underwent fast freezing (Plasma Blast freezer, Harris-Revco, Cendro, Asherville, NC) at –35°C and were kept at this temperature for approximately 2 months, at which point they were shipped to our medical center, where they were kept at –80°C until use (up to 6 months).

Plasma thawing and sample preparation

The 20 FFP units were thawed (Helmer Plasma Thawing System DH8, Noblesville, IN) at 37°C within 16 minutes. Aliquots were taken for measurement of coagulation factors. The plasma units were then kept for 24 hours at 4°C. At this second time point, aliquots were taken again from the FFP bags, refrozen and kept at –80°C. One week later the above procedure was repeated.

Coagulation factor measurements

Coagulation factors, prothrombin, FVII, F IX, and FX, were measured immediately after sampling from the transfusion bags. Coagulation-factor activity was measured by a coagulation analyzer (Coagulation Analyzer, SYSMEX CA 1500, Dade Behring, Kobe, Japan). Specific factor-deficient plasma (Biopool International, Ventura, CA) was utilized as a test reagent for each factor. Standard human plasma (Dade Behring, Newark, NJ) was used as a reference to construct a standard curve, and factor levels of diluted samples were calculated from these curves. A normal control plasma (Control Plasma N, Dade Behring) and a pathologic control (Control Plasma P, Dade Behring) was used for each specific factor.

Fibrinogen measurements

Fibrinogen level was measured by the coagulation analyzer (SYSMEX CA 1500) using Thrombin (Dade Behring) as a catalyzer. Normal plasma (Ci Trol, level 1, Dade Behring) was utilized to construct a standard curve, and factor levels of diluted samples were calculated from the curve. Ci Trol level 2 (Dade Behring) was employed as a control of abnormal value.

Statistical analysis

Statistical analyses and all computations were done using a statistical software package (the SPSS version 10, SPSS Inc., Chicago, Illinois).[13–15] Graphs were drawn using a statistical software package (S-plus version 4.5, MathSoft, Seattle, WA). One way ANOVA tests were used to examine differences between blood groups at each time point. One-sample *t* test was employed to examine the assumption that at each time point the mean of each coagulation-factor level will be above its normal lower limit. The reason for using a one-sample *t* test is that, having a defined target value, the lower limit of normal, and comparing the result of our sample to the given value, the target value can be considered as the "population parameter" while the computed sample value is the "estimate." The probability of the estimate is tested (the p-value) against the given parameter. The method of doing so is the one-sample *t* test.

For each of the factors at the four time points, a 95-percent CI was calculated to assess the assumption that the mean result is significantly within the lower and upper normal limits.

The basic assumption for statistical evaluation was that, for each factor and each time point, the mean result is equal to or less than the lower limit of normal, while the alternative is that the mean result is greater than the lower limit.

RESULTS

The levels of all 20 FFP units at Time 0 of the first thaw was above the lower value of normal for prothrombin, FVII, F IX, FX, and fibrinogen except for one FFP unit (group AB) that had 61.3-percent activity of FVII (normal values, 63–139%).

Figure 1 depicts the mean levels of all factors measured by blood group (A, B, AB, and O), along with the mean for all 20 FFP units for each of 4 time points: immediately after the first thaw, at 24 hours of 4°C, immediately after the second thaw, and 24 hours thereafter. As can be

seen from the curves, the mean levels do not vary greatly between blood groups. FVII, F IX, FX, and fibrinogen remained within both limits of normal for all blood groups, while the level of prothrombin was found to be above the upper limit of normal for five FFP units of blood group B only.

These possible differences between blood groups were examined for each of the five factors at each time point, and the p values for each factor at four time points were found to be not significant, except for FVII at the first two time points, as can be seen in Table 1.

Based on these results, the remaining data analysis was performed on all 20 FFP units as one group, regardless of blood group.

Table 2 presents the mean ± SD levels of each coagulation factor examined at each time point. As can be seen, all levels were above the lower and actually within both limits of normal, as demonstrated by 95-percent CI values (data not shown) for Time 0, immediately after the first thaw, after 24 hours at 4°C, at the second thaw, or after 24 hours at 4°C of the second thaw. The p values for all the means in the table (each one against its lower limit) are less than 0.005, implying that the mean results are significantly above the lower normal limits (see "Statistical analysis").

Looking at factor levels at Time 0, immediately after the first thaw, compared to the last time point, after 24 hours at 4°C after the second thaw, one can see that the mean levels of all factors hardly changed: prothrombin from 114 to 112 percent (2%), FVII dropped from 92 to 82 percent (11%), F IX from 95 to 92 percent (3%), FX from 111 to 104 percent (6%), and fibrinogen changed from 296 to 302 mg per dL (2% increase).

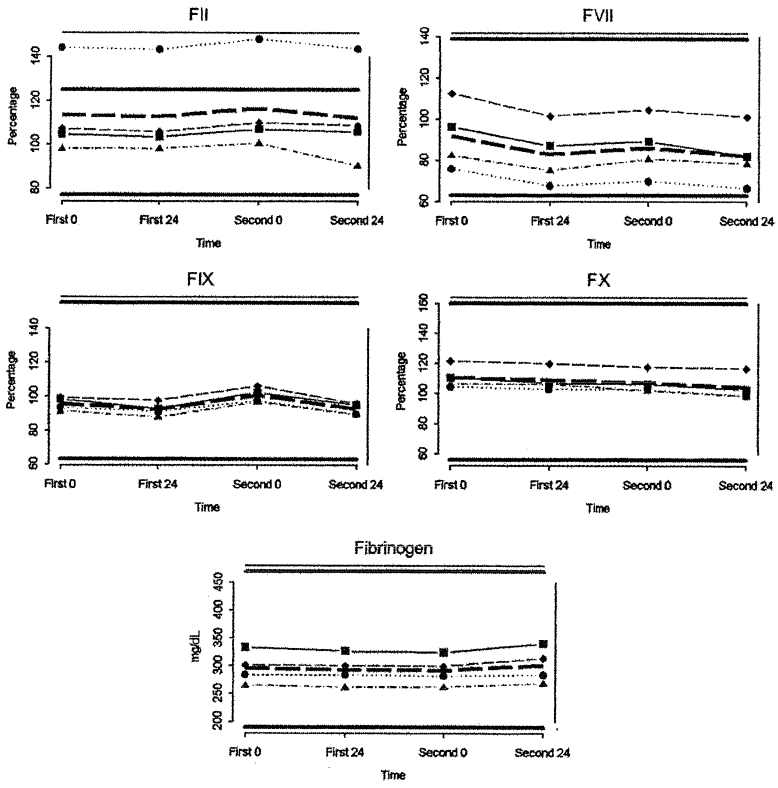

Fig. 1. Mean level of coagulation factors for FFP group A (-■-), B (···●···), AB (-·-▲-·-), O (—◆—); all 20 FFP units (——) normal values (——).

TABLE 1. Differences (p values) between blood groups for each factor at four time points

Factor	First thaw Time 0	First thaw Time 24	Second thaw Time 0	Second thaw Time 24
FII	0.578	0.584	0.536	0.488
FVII	0.019	0.027	0.067	0.262
F IX	0.847	0.816	0.598	0.624
FX	0.491	0.488	0.452	0.543
Fibrinogen	0.139	0.216	0.199	0.122

DISCUSSION

We examined whether the levels of vitamin K-dependent clotting factors and fibrinogen in twice-frozen-and-

TABLE 2. Mean ± SD level of coagulation factors at all time points

Coagulation factor	Normal limits	First thaw Time 0	First thaw Time 24	Second thaw Time 0	Second thaw Time 24
Prothrombin (%)	77–125	114 ± 55	113 ± 55	116 ± 54	112 ± 54
FVII (%)	63–139	92 ± 21	83 ± 20	86 ± 22	82 ± 28
F IX (%)	63–155	95 ± 15	92 ± 15	101 ± 12	92 ± 10
FX (%)	56–160	111 ± 19	109 ± 18	107 ± 17	104 ± 22
Fibrinogen (mg/dL)	190–470	296 ± 49	293 ± 50	292 ± 48	302 ± 52

thawed (i.e., four time points) FFP of blood groups A, B, O, and AB remain stable, above the lower and within both normal limits. The hypothesis tested was that the levels may decrease, thus precluding the use of this component for transfusion,

It was found that the activity of all factors examined remained above the lower normal limit at all four time points, regardless of blood group (Fig. 1).

FVII, F IX, FX, and fibrinogen remained within both normal limits for all blood groups, whereas the level of prothrombin was found to be above the upper limit of normal for five FFP units of blood group B only, even at Time 0 of the first thaw (Fig. 1), albeit without significant difference ($p > 0.05$) in relation to other blood groups (Table 1). We do not yet have an explanation for this observation and it merits further investigation. It may be, however, that levels of prothrombin in group B individuals are normally higher, much as the levels of FVIII:C and vWF are lower in normal individuals with blood group O[10,16,17] or secretors of Lewis antigen;[18] or like the observation that group AB cryo-supernatant contains lower levels of fibrinogen, FV, FVIII, and vWF:Ag than groups O or B.[19]

Looking at variations among blood groups, it was found that for FVII there was some variation among blood groups (Fig. 1), that even reached borderline significance ($0.01 < p < 0.05$) for Time 0 and 24 hours after the first thaw (Table 1). However, this significance was lost at time points 3 and 4, which may suggest that it could be attributed to statistical chance. It should be taken into consideration that the size of the study and the range of FVII concentrations may contribute to this statistical chance as well.

Therefore, given that all mean levels fell above the lower limits of normal for all factors at all time points, the rest of the data analysis was performed on all 20 FFP units of all blood groups combined together.

Taken together, the data reveals that the levels of prothrombin, FVII, F IX, FX, and fibrinogen remain within normal limits after FFP is thawed and refrozen twice and kept twice at refrigerator temperature.

Processing whole blood to plasma for transfusion involves a number of steps that can affect the stability of coagulation factors; many of these steps have been examined. The time elapsing from collection of the unit to separation and freezing of plasma was examined, and it was concluded that most factors (except for FVIII) remain stable if separation and freezing occur within 24 hours of collection.[8] The stability of clotting factors for different lengths of time at –20°C, –40°C,[20] and –65°C was tested, and it is now accepted that the latter can be stored for up to 7 years.[1] The activity of clotting factors, including vitamin K-dependent proteins[10] and fibrinogen[21] after thawing at different conditions[22,23] were also investigated. Methods of freezing and the rapidity of freezing and thawing, such as microwave versus waterbath,[24–28] were examined.

The only data about the fate of coagulation proteins in plasma after repeated freezing and thawing concerns FV and FVIII:C,[12] where it was observed that the activity of FVIII:C decreased by 25 to 35 percent.

Our results, addressing the issue for the first time, have shown that the levels of prothrombin, FVII, F IX, FX, and fibrinogen were changed between +2 percent and –11 percent upon twice freezing and thawing, and remained above the lower limit of normal at all time points. This suggests that twice-frozen plasma can be used safely for transfusion and can be especially useful for rare donors or unused autologous plasma.

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