Original Article

The effect of repeated freezing and thawing on levels of vitamin K-dependent coagulation factors and fibrinogen in fresh frozen plasma

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Abstract:

Background: Fresh frozen plasma (FFP) is considered adequate for transfusion immediately after thawing or for up to 24 hours if kept at 1–6°C, and is currently used very often to replace deficient clotting factors. If factor levels in refrozen FFP are within normal limits, then this component can possibly be transfused, thus avoiding wastage of FFP. **Aim:** To study the fate of vitamin K-dependent coagulation factors (F II, F VII, F IX, F X) and fibrinogen activity levels in repeatedly (twice) frozen and thawed FFP. **Materials and Methods:** Two hundred FFP units comprising 50 units of each major blood group (A, B, AB, and O) were thawed at 37°C and 10–20 mL of FFP transferred to transfer bags with the help of a sterile connecting device (SCD). The FFP samples were taken into tubes (first sampling), and then the transfer bags were kept for 24 hours at 4°C. After 24 hours, repeat samples were taken in tubes from the transfer bag (second sampling), and then the bags were re-stored at < -18°C. One week later, the above procedure was repeated. Activity of coagulation factors and fibrinogen levels were measured by the automated coagulation analyzer. **Results:** The levels of F II, F VII, F IX, F X, and fibrinogen of all the 200 FFP units, at all four time points, were above the lower normal value, but well within the normal range. **Conclusion:** The levels of F II, F VII, F IX, F X, 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.

Key words:

Fresh frozen plasma, freezing and thawing, vitamin K-dependent clotting factors

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Introduction

Component therapy has had a profound impact on the practice of transfusion medicine. When only the component that is needed is transfused, the patient is spared untoward effects of other blood components.

When FFP is frozen within eight hours of its preparation at < -18°C, levels of coagulation factors like F V, F VIII, F II, F IX, F X, F XI, and fibrinogen remain stable with good relevant activity.[1]

FFP is considered adequate for transfusion immediately after thawing or for up to 24 hours if kept at 1–6°C. FFP is used as a hemostatic support either for treatment or for prophylaxis.[2] FFP should not be used as a volume expander or as a nutritional source.[3]

Materials and Methods

Preparation and storage of FFP

Two hundred FFP units comprising 50 units of each major blood group (A, B, AB, and O) were prepared from whole blood collected from healthy voluntary donors in CPDA-1 (CPDA-1: citrate phosphate dextrose adenine) multiple bags. Separation of plasma was performed by two centrifugation steps and the FFP units thus formed were stored at less than -18°C [as per the guidelines of the American Association of Blood Banks (AABB)]. The study period was from January 2010 to May 2011.

Plasma thawing and sample preparation

The FFP bags were thawed at 37°C immediately after taking out from the deep freezer (-18°C) within 24 hours of preparation, that is, day 0. Approximately 10–20 mL FFP (representative samples) was taken in transfer bags with the help of a sterile connecting device (SCD), and then for assessment of various coagulation factors, 5 mL sample (first sampling) was taken into the test tube. The FFP transfer bags were then kept for 24 hours at 4°C after the sampling. At the end of 24 hours, repeat samples were taken in test tubes from the above FFP transfer bag (second sampling). After the repeat sampling, the transfer bags were re-stored at less than -18°C. One week later, the above procedure was repeated.

Measurement of vitamin K-dependent coagulation factors (FII, FVII, FIX, and FX), and fibrinogen

Coagulation factors were measured immediately in the test tubes sampled from the transfer bags. Activity of the coagulation factors was measured by a fully automated coagulation analyzer (Stago Compact, France). Specific factor-deficient plasma was utilized as a test reagent for each factor (as per the instructions of the manufacturer). Standard human plasma (system control N+P, Stago) was used as a reference to construct a standard curve and factor levels of diluted samples were calculated from these curves. System control negative + positive (N + P) (Stago) was employed as a control for abnormal values.

Evaluation and statistical analysis of readings

The levels of vitamin K-dependent coagulation factors (FII, FVII, FIX, and FX), and fibrinogen in all the 200 bags of FFP used in this study were compared as per the values obtained on day 0 and 7. The results were evaluated and further analyzed by statistical methods for any significance by using Epi-info software version 2011.

Results

The purpose of this study was to measure the levels of vitamin K-dependent coagulation factors (F II, F VII, F IX, and F X), and fibrinogen of 200 FFP units at four time points (freezing and thawing twice), that is, the first time point was immediately after the first thaw. The second time point was after keeping this thawed FFP for 24 hours at 4°C. The third time point was immediately after the second thaw. The fourth time point was after keeping this thawed FFP again for 24 hours at 4°C. The normal values of vitamin K-dependent coagulation factors are as follows: F II: 70–120%; F VII: 55–170%; F IX: 60–150%; F X: 70–120%, and fibrinogen 200–400 mg/dL. The levels of F II, F VII, F IX, F X, and fibrinogen of all 200 FFP units, at all four time points, were above the lower normal value, but well within the normal range.

To make a comparison of the values of the vitamin K-dependent coagulation factors and fibrinogen, at all four time points, 50 FFP units were taken from each blood group A, B, AB, and O. Figures 1a-e depict the mean levels of all factors measured

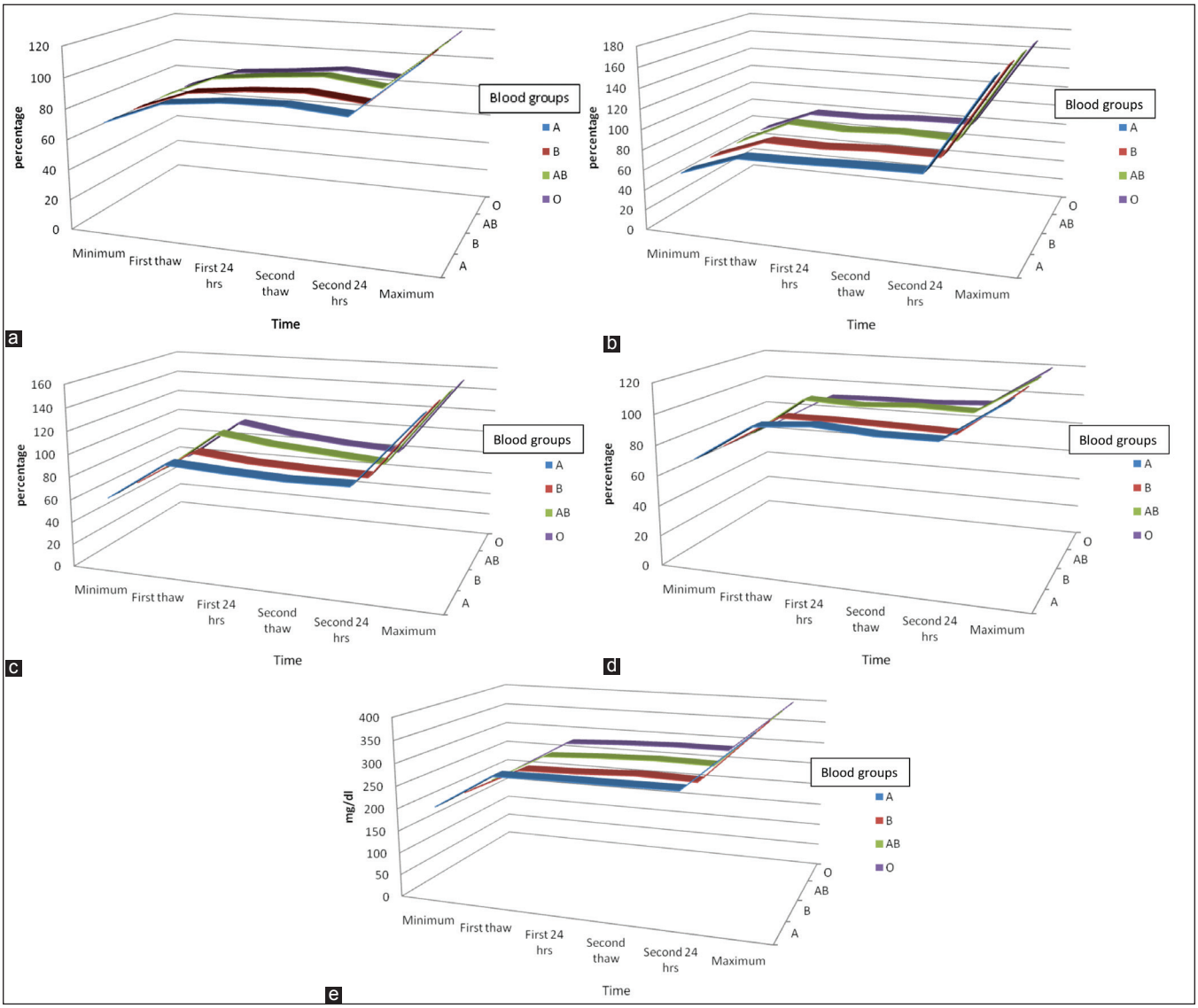

Figure 1: (a) Mean level of coagulation factor II for all 200 FFP units (b) Mean level of coagulation factor VII for all 200 FFP units (c) Mean level of coagulation factor IX for all 200 FFP units (d) Mean level of coagulation factor X for all 200 FFP units (e) Mean level of coagulation factor Fibrinogen for all 200 FFP units

according to the blood groups (A, B, AB, and O) of FFP units for each of the four time points. As can be seen from the graphs, the mean levels do not vary greatly between the blood groups. F II, F VII, F IX, F X, and fibrinogen remained within the limits of normal for all blood groups.

Table 1 represents the differences (*p* values) between blood groups of each factor at four time points. It shows that all the results (*p* values) were not significant statistically (>0.05) except for F II at second thaw (time 0) (0.009) and for F IX at first thaw (time 0) (0.017).

Table 2 represents the mean ± standard deviation (SD) levels of each coagulation factor examined at each time point. As can be seen, all levels were above the lower limit and actually within both limits of normal, as demonstrated by 95% confidence interval (CI) values for all four time points. The *p* values for all the means in the table (each one against its lower limit) are 0.000005, implying that the mean results are highly significant.

Comparing the factor levels immediately after the first thaw (time 0) with those after the second thaw (time 24), one can see that the mean levels of all factors hardly changed, and if changes were there, they were not statistically significant: F II changed from 84 to 87% (3% increase), F VII changed from 77 to 79% (2% increase), F IX dropped from 95 to 82% (13%), F X remain unchanged (93%), and fibrinogen changed from 271 to 278 mg/dL (7% increase).

Discussion

FFP is given primarily for two indications, to prevent bleeding (prophylaxis) or to stop bleeding (therapeutic). Prophylactic transfusions, which may account for over 50% of all FFP transfusions, are largely given before surgery or an invasive procedure.[4]

Recommendations for the transfusion of FFP have remained relatively consistent over the years. These include 1) active bleeding, or before surgery or an invasive procedure in patients (adults and neonates) with acquired deficiencies of one or more coagulation factors as demonstrated by an increased international normalized ratio (INR), prothrombin time (PT), or activated partial thromboplastin time (aPTT), when no alternative therapies are available or appropriate; 2) immediate correction of vitamin K deficiency or reversal of warfarin effect in a patient with active bleeding, or before surgery/an invasive procedure (in conjunction with use of prothrombin complex concentrates); 3) disseminated intravascular coagulation (DIC) or consumptive coagulopathy with active bleeding; 4) thrombotic thrombocytopenic purpura (TTP); and 5) active bleeding, or before surgery/an invasive procedure in patients with a congenital factor deficiency when no alternative therapies are available or appropriate.[5] Previous common uses of FFP that are now considered inappropriate include volume replacement, correction of hypoalbuminemia, nutritional support, and immunoglobulin replacement because safer alternatives (colloids and crystalloids) are available.[5]

The liver is the major site of synthesis of coagulation factors II, V, VII, IX, X, XI, XII, and fibrinogen as well as of factors with potential antithrombotic activity such as protein C, S, and antithrombin III. Patients with severe liver disease may experience defects in factor synthesis and increased factor degradation that can result in generalized bleeding. Prolongations of PT and aPTT are the most frequent abnormalities among the commonly performed clotting tests in patients with liver disease, and may reflect impaired protein synthesis, deficiency of vitamin K, or even DIC. The presence of an abnormal test does not necessitate intervention, especially in the nonbleeding patient. The routine use of FFP as prophylaxis for excessive surgical bleeding in patients with severe liver disease finds few supporters and less evidence of benefit.[6]

An acquired deficiency of factors II, VII, IX, and X occurs due to treatment with anticoagulants like warfarin. So, FFP is one of the major indications for warfarin reversal. The major risk of anticoagulant therapy is hemorrhage. For patients treated with the oral vitamin K antagonists, the annual risk of severe hemorrhage ranges from 1 to 5%.[7] Warfarin is an oral anticoagulant used for the prevention of recurring thrombosis. It works by blocking a hepatic carboxylation step for the vitamin K-dependent coagulation factors II, VII, IX, and primarily, X. This effect is reversed by the administration of vitamin K, and monitored most commonly by INR, which is calculated from the prothrombin time and standardized across laboratories.[8]

Table 1: Differences (*p* values) between blood groups for each factor at four time points

Coagulation factor	First thaw (time 0)	First thaw (time 24)	Second thaw (time 0)	Second thaw (time 24)
Factor II	0.217	0.265	0.009	0.134
Factor VII	0.176	0.609	0.526	0.351
Factor IX	0.017	0.150	0.062	0.533
Factor X	0.067	0.295	0.295	0.136
Fibrinogen	0.215	0.156	0.334	0.123

p value =0.05 (significant)

Table 2: Mean ± SD level and 95% CI of coagulation factors at all time points

Coagulation factor	Normal limits	First thaw (time 0)	95% CI	First thaw (time 24)	95% CI	Second thaw (time 0)	95% CI	Second thaw (time 24)	95% CI
Factor II (%)	70–120	84±8	76–92	88±10	78–98	91±5	86-96	87±7	89–94
Factor VII (%)	55–170	77±14	63–91	77±14	63–91	79±13	66-92	79±14	65–93
Factor IX (%)	60–150	95±12	83–107	90±9	81–99	86±8	78-94	82±5	77–87
Factor X (%)	70–120	93±14	79–107	94±15	79–109	94±12	82-106	93±14	79–107
Fibrinogen (mg/dL)	200–400	271±42	229–313	276±39	237–315	279±37	242-316	278±36	242–314

p value =0.000005 [analysis of variance (ANOVA) test] SD: Standard deviation; CI: Confidence interval

There are two main possible scenarios for the use of FFP in patients taking warfarin: The first scenario is nonbleeding patients with elevated INR. This scenario does not usually require transfusion of FFP. In nonbleeding patients with INR up to 9.0, the management protocols are to hold warfarin dose and give oral vitamin K if INR >5. In this scenario, FFP is not indicated. In nonbleeding patients with INR >9.0, the protocol is to hold warfarin dose and give a higher dose of oral vitamin K. In this scenario too, FFP is not indicated.[8]

The second scenario is in cases of bleeding patients at any INR. Here it is advised to hold warfarin dose, administer vitamin K, and transfuse FFP. FFP is transfused in bleeding patients, regardless of the INR. The use of FFP in this scenario is pretty well accepted, but FFP may not be the best product to use. Alternatives of FFP are prothrombin complex concentrate (PCC) and recombinant activated factor VII (NovoSeven). Vitamin K should be administered regardless of the use of FFP. FFP administered at a dose of 10–20 mL/kg of body weight increases factor levels by 20–30%.[8] Frequency of transfusion depends on the half-life of the deficient factor/factors. In adults and big children, dosing is rounded to the nearest number of units. Number of units = desired dose (mL)/200 mL/unit.[5]

As per the guidelines of the AABB, once FFP is thawed, it must be stored at 4 ± 2°C for no longer than 24 hours before infusion. FFP must not be refrozen, but once thawed (or after one year of storage and thaw), it can be used as single-donor plasma. This single-donor plasma will replace only stable coagulation factors and it can be stored up to five weeks.[9]

The concentration of coagulation factor, the citrate concentration, and the volume of each unit may vary depending on the characteristics of the donor and of the collection.[9] Processing whole blood to plasma for transfusion involves a number of steps that can affect the stability of the coagulation factors. Many of these steps have been examined. The time elapsing from the collection of the unit to separation and freezing of plasma have been examined, and it was concluded that most factors (except F VIII) remain stable if separation and freezing occur within 24 hours of collection.[10] The composition of FFP can be influenced by many factors. These would include gender and age and genetic, dietary, and other environmental factors, all potentially modifying the levels of individual proteins. The composition of collected plasma would also be affected by the processing procedure, including how quickly the plasma is collected and then stored. Although clinicians tend to assume approximate equivalence in clinical effectiveness between individual units of FFP, it is likely that there is individual variation. This variation reflects not only the biological differences in constituents between donors (for example, von Willebrand factor and F VIII levels, being ABO dependent), but also differences in processing, storage, and preparation for administration. Such variations might be expected to be less marked for pooled plasma components such as solvent/detergent-treated FFP.[5]

The stability of clotting factors for different lengths of time at -20, -40,[11] and -65°C was tested, and it is now accepted that the latter can be stored for up to seven years.[3] The activity of clotting factors, including vitamin K-dependent proteins[12] and fibrinogen[13] after thawing at different conditions[14,15] has also been investigated. Methods of freezing and the rapidity of freezing and thawing, such as microwave versus water bath,[16-18] have been examined. However, there has been just one study in which the vitamin K-dependent coagulation factors and fibrinogen have been assessed after twice-freezing and twice-thawing.[2]

The only data about the fate of coagulation proteins in plasma after repeated freezing and thawing concerned the fate of labile coagulation factors F V and F VIII:C and were by Dzik,[19] where it was observed that the activity of F VIII:C decreased by 25–35%.

In this study, the main aim was to evaluate whether the levels of vitamin K-dependent clotting factors and fibrinogen on twice-frozen-and-thawed (that is, four time points) FFP of blood groups A, B, AB, and O remained stable, above the lower limit and within both normal limits. It was found that the activity of all factors examined remained above the lower normal limit at all four time points, regardless of the blood group [Figures 1a-e].

This study reflected that F II, F VII, F IX, F X, and fibrinogen remained within both normal limits for all blood groups. A similar study done by Ben–Tal *et al.*[2] showed that all these factors remained within both normal limits for all blood groups except for FII. In the study of Ben–Tal *et al.*, the levels of prothrombin was above the upper limits of normal for blood group B only, even at time 0 of first thaw, but that was also not statistically significant in relation to other blood groups.[2] They do not have any explanation for this observation but the possible explanation is that the levels of prothrombin in individuals with B blood group are normally higher. Some studies have evidence that the levels of FVIII:C and vWF are lower in normal individuals with blood group O. Secretors of Lewis antigens and group AB cryosupernatant contain lower levels of fibrinogen, F V, F VIII, and vWF:Ag than group O or B.[20]

In this study, it was found that there were no variations among blood donors of different blood groups [Figures 1a-e]. There were some values which showed statistical significance (p <0.05) for F II at second thaw (time 0) (p =0.009) [Table 1]. However, this significance was lost at time point 4. Similarly, values of F IX at first thaw (time 0) showed statistical significance (p =0.017) [Table 1]. However, this significance was also lost at time points 2, 3, and 4. Though the discrepancies noted here are statistically significant values, the possible explanation one can think of is because even a calibrated automated machine may give different result values for the same sample processed at different times.

The study results have shown that the levels of prothrombin, F VII, F IX, F X, and fibrinogen were changed between + 2 and -13% upon freezing and thawing twice and remained above the lower limit of normal at all time points.

Taken together, the data reveals that the levels of prothrombin, F VII, F IX, F X, and fibrinogen remain within normal limits after FFP was thawed, kept at refrigerator temperature 2–6°C for 24 hours, refrozen, and thereafter kept again at refrigerator temperature 2–6°C for 24 hours.

Conclusion

The study concludes that on repeated freezing and thawing of FFP, the levels of vitamin K-dependent coagulation factors and fibrinogen remain within the normal range and possibly without

losing their functional ability. Once FFP is thawed and the treating physician or surgeon is unable to transfuse the same within 24 hours, then the component is usually discarded. In large surgical and trauma centers, discarding of these unused FFP units creates a significant waste of resources. In special circumstances like rare donors, unused autologous plasma, and postponement of surgery due to any reason, these FFP units can be refrozen and used again at the time of surgery.

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