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Effects of Different Quick-Freezing Methods and Storage Temperatures on the Content of Cryoprecipitated Blood Coagulation Factors in Fresh Plasma

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ABSTRACT

To observe the effect of different quick-freezing methods and different storage temperatures on the content of cryoprecipitated blood coagulation factors in fresh plasma. The convenient sampling method was used to select 200 ml of whole blood from 60 unpaid blood donors who entered the blood station from January 2022 to October 2022. The acquisition time is controlled within 10 min. The plasma samples were divided into six groups (A, B, C, D, E, F) by using a random number table, 10 in each group. Group A (plate freezer for 30 min, - 30 ℃ storage), Group B (plate freezer for 30 min, 4 ℃ storage), Group C (plate freezer for 120 min, - 30 ℃ storage), Group D (plate freezer for 120 min, - 4 ℃ storage), Group E (plate freezer for 30 min, - 4 ℃ storage), Group F (plate freezer for 120 min, 4 ℃ storage). At 0, 3, 5, 7 and 9 h after the completion of the preparation of cryoprecipitate, the changes and differences of the content of coagulation factor VIII (Fv III) and fibrinogen (FIB) in the six groups of samples were observed. The highest level of FvIII occurred in group B at 5 h with a concentration of (2.47±0.56) IU/mL, while the lowest level occurred in group A at 9 h with a concentration of (0.84±0.07) IU/mL. The highest level of FIB occurred in group A at 0 h with a concentration of (245.56±25.92) mg/200ml, while the lowest level occurred in group C at 9 h with a concentration of (195.16±13.98) mg/200ml. It is preferable that both FvIII and FIB levels are at a higher level at 9 h, with group B having more appropriate levels of FvIII and FIB. According to the analysis of variance, the content of FvIII will be affected by the storage temperature, time, quick-freezing method, and the interaction between quick-freezing method and time ($P<0.05$). The content of FIB will be affected by the effects of quick-freezing method, time, quick-freezing method and storage temperature ($P<0.05$). Different quick-freezing methods and storage temperatures affect FvIII and FIB in the cryoprecipitated fresh plasma. The optimal quick-freezing method is the plate quick-freezing machine for 30min, and the storage temperature is 4℃. Under this condition, the content of coagulation factor in the cryoprecipitate decreased more slowly.

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HW, ZY and KL conceived the research idea and designed the study. HW, NZ and KL visited the dairy farms and collected the data. HW and KL performed data analysis. All authors discussed the results and wrote the manuscript.

Key words
Fresh plasma, Coagulation factor, Quick-frozen, Preservation, Fibrinogen

INTRODUCTION

Although there are guidelines for the industrial manufacture of plasma-derived drugs, there is no definitive recommendation for the use of plasma for fractionation as there is unknown impact on the activity of clotting factors with prolonged storage (Politou *et al*., 2022). The Blood and Biologics Development Association and the Food and Drug Administration require that the production of cold precipitate must complete its freezing process within 8 h (Lokhandwala *et al*., 2022). Cold precipitate, a white flocculent substance formed during the freezing of whole blood preparations, contains various blood components such as clotting factors, fibrinogen, and others that can be used to treat diseases such as hemophilia and fibrinogen deficiency. Transfusion is an emergency measure prepared for trauma patients, which begins with thawing of plasma, and the usage process must be completed within 24 h after plasma thawing. As time passes, the levels of clotting factors VIII (FvIII) and fibrinogen (FIB) in plasma cold precipitate will decrease (Wang *et al*., 2014). In China, the individual differences in fresh frozen plasma are mainly due to FVIII (Zhu *et al*., 2019). Meanwhile, there is also a certain amount of FIB in frozen precipitate, and the level of FIB directly determines the plasma's suitability for bleeding patients (Thomas *et*

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al., 2021). It can be seen that the presence of FvIII and FIB in cold precipitate has become an important factor affecting the storage of fresh frozen plasma.

China's quality requirements for whole blood and component blood (GB18469-2012) points out that when collecting fresh frozen plasma, the whole blood should be stored in a cryogenic environment. The cryoprecipitate in plasma should be separated within 18 h and the component blood in solid state should be frozen quickly. The 13th edition of the American Blood Bank Association standard stipulates that the treated coagulation factor VIII (Fv III) can be stored for 6 h, and the limit of storage temperature is not stipulated (Spivey *et al*., 1992). Due to the geographical location of the collection points of unpaid blood donation, it is still necessary to return to the central blood station to prepare fresh frozen plasma after the whole blood collection. Therefore, the acquisition time is limited. The coagulation factor III (FvIII) contained in the cryoprecipitate is unstable and has a half-life of 8~12h, so the biological activity is easy to lose. Many links, including whole blood preparation, fresh plasma preparation, and cold precipitation affect the coagulation factor VIII *in vitro*. Yang *et al*. (2006) found that the activity of FVIII was (100.51±44.02%) at 0 h and gradually decreased, particularly affected by the rapid freezing time and storage temperature. Piedras *et al*. (1993) have found that plasma freezing temperature (- 70℃, -30 ℃), anticoagulant, storage time, etc. affect the activity of FVIII in cold sediment. Therefore, the purpose of this study is to explore the effects of different quick-freezing methods and storage temperatures on the content of cold-precipitated coagulation factors in fresh plasma.

MATERIALS AND METHODS

Sample source

The convenient sampling method was used to select 60 unpaid blood donors who participated in the blood station from January 2022 to October 2022, each with 200 ml of whole blood. All unpaid blood donors meet the requirements for health examination of blood donors (GB18467-2011). Sample collection shall be completed within 10 min. After collection, 6 groups of whole blood were prepared with ACD-B and CPDA-1 as preservation solution. After separation and preparation of cold precipitate, cold precipitate detection was immediately performed at 0h, 3h, 5h, 7h, and 9h after blood collection. Based on the fact that A, B, and AB blood types contain higher levels of clotting factors, adult male O blood type was chosen as the sample source to ensure the balance of baseline data in this study.

Instruments and reagents

Instruments include desktop low-speed centrifuge (Kubota Company, Japan); Sanyo Cryogenic Refrigerator (Sanyo Electric Co., Ltd.), 6000i large-capacity cryogenic centrifuge (Thermo Fisher), German TECO Coatron1800 Automatic Blood agglutator (TECO), Constant temperature water bath (Shenzhen Dako for Co., Ltd.).

Reagents include F VIII reagent kit (produced by TECO), FIB reagent kit (produced by TECO).

Preparation method

The plasma was centrifuged for the first time within 6 h. The centrifugal parameter was 3400 r/min, 12 min. The collection temperature was set at 4 ℃, and 200 ml of fresh plasma was prepared. The samples were divided into six groups: A, B, C, D, E and F. Group A was quick-frozen by the plate quick-freezer. The quick-freezing time was set at 30min. After that, the group was put into a low temperature box at - 30 ℃ for cold storage. After 3 days, it was thawed in a low temperature thawing box, and then the whole blood was centrifuged at 4000r/min for10min. After centrifugation, remove the upper plasma visible to the naked eye, extract white flocs, and obtain 20~30ml of cold precipitation. The cold precipitates were distributed to test tubes 0~4, 4~6ml each. The time when the cold precipitation preparation is completed is regarded as the time zero. At this time, immediately take the test tube 0 to test the content of coagulation factor VIII (Fv III) and fibrinogen (FIB) and time it. Use the No. 1 test tube as the test sample at the time of 1 day after 0:00, and so on. The content of Fv III and FIB were measured at 0, 3, 5, 7, 9 days respectively. Group B changed the rapid freezing time to 30 min and stored it at 4℃, before being thawed using a low-temperature thawing box after 3 days. Group C changed the rapid freezing time to 120 min and stored it in a -30℃ freezer before being thawed using a low-temperature thawing box after 3 days. Group D changed the rapid freezing time to 120 min and stored it in a -4℃ freezer before being thawed using a low-temperature thawing box after 3 days. Group E stored the plasma at -4℃ and thawed it using a low-temperature thawing box after 3 days. Group F changed the rapid freezing time to 120 min and stored it at 4℃, before being thawed using a low-temperature thawing box after 3 days. Group A was marked as A_0, A_1, A_2, A_3, A_4; Group B was marked as B_0, B_1, B_2, B_3 and B_4 respectively; Group C was marked as C_0, C_1, C_2, C_3, C_4; Group D is marked as D_0, D_1, D_2, D_3 and D_4, respectively; Group E was marked as E_0, E_1, E_2, E_3 and E_4, respectively; Mark group F as F_0, F_1, F_2, F_3 and F_4, respectively.

Observation indicators

The cold precipitate clotting factor VIII and FIB levels of each group sample were compared at 0h, 3h,

5h, 7h, and 9h. Prior to each test, a visual inspection was performed, and all samples were thawed at 37°C water bath. The appearance of the cold precipitate was determined to be qualified by observing that it appeared pale yellow and had no color abnormalities and that it was clear and free of fibrinogen precipitation. The clotting method was used to determine the levels of FvIII and FIB, and 1.5ml of sample was taken for the relevant measurements. The evaluation criteria for FvIII and FIB were based on the "Quality Requirements for Whole Blood and Blood Components (GB18469-2012)," where FvIII ≥ 0.200 IU/mL whole blood and FIB ≥ 75 mg/200mL whole blood were considered qualified (Zhou, 2013).

Statistical analysis

SPSS 26.0 software is used for data analysis. The measurement data in line with the normal distribution is expressed by (x ± s), and one-way ANOVA is used. The comparison between the two was carried out by kruskal-wallis test. Different quick-freezing methods and different storage temperatures are compared using factorial design analysis of variance, and counting data were used χ^2 Inspection and calibration level α= 0.05. All of them are considered statistical difference with P<0.05.

RESULTS

Comparison of the content of cryoprecipitated coagulation factor III between different groups

The content of FvIII in different groups at different time points is shown in Table I. The highest content of FvIII appeared at 5h in Group B, and the lowest at 9h and 0h in Group A. There was no significant difference between the groups (P>0.05). At 3h, only group C was lower than group E, and there was a significant difference (P<0.05). At 5h and 7h, group A was significantly lower than other groups (P<0.05). At 7h, group B was lower than group C, D and E (P<0.05). At 9h, group A was lower than group D and E (P<0.05). The lowest values of FvIII levels at 0h, 3h, 5h, 7h, and 9h were found in groups F (1.94±0.38) IU/mL, E (1.76±0.69) IU/mL, A (1.42±0.39) IU/mL, A (0.88±0.32) IU/mL, and A (0.84±0.07) IU/mL, respectively. The highest values of FvIII levels were found in groups E (2.42±0.48) IU/mL, C (2.41±0.65) IU/mL, B (2.47±0.56) IU/mL, C (2.02±0.57) IU/mL, and E (1.33±0.35) IU/mL at 0h, 3h, 5h, 7h, and 9h, respectively. At 9h, the FvIII levels were at a higher level, and groups D and E could be considered as alternatives (Table I).

Comparison of FIB content of cold precipitation between different groups

See Table II for FIB content between different groups at different time points. From Table II, the highest content of FIB appeared at 0h in Group A and the lowest at 9h in Group C. At 0h, there was only significant difference between group A and group B, C and D (P<0.05). At 3h, there was no significant difference between the groups (P<0.05). At 5h, there was only significant difference between group A and other groups (P<0.05). At 7h, group A and B were higher than group C, D and E (P<0.05). At 9h, group A was higher than group D and E (P<0.05). The lowest values of FIB levels at 0h, 3h, 5h, 7h, and 9h were found in groups B (222.88±24.66) mg/200ml, D (210.51±27.01) mg/200ml, F (220.93±18.56) mg/200ml, C (199.69±26.48) mg/200ml, and C (195.16±13.98) mg/200ml, respectively. The highest values of FIB levels were found in groups A (245.56±25.92) mg/200ml, E (233.87±21.17) mg/200ml, A (220.93±18.56) mg/200ml, A (214.73±16.40) mg/200ml, and B (207.18±15.23) mg/200ml at 0h, 3h, 5h, 7h, and 9h, respectively. At 9h, the FIB levels were at a higher level, and groups A, E, and F could be considered as alternatives (Table II).

Table I. Comparison of Fv III content between different groups (IU/mL, x ± s).

Groups (n)	0h	3h	5h	7h	9h
A(10)	1.99±0.54	2.16±0.59	1.42±0.39^{*bcdef}	0.88±0.32^{*bcdef}	0.84±0.07^{*de}
B(10)	2.27±1.08	2.27±0.63	2.47±0.56^{a}	1.43±0.50^{*acde}	1.28±0.21*
C(10)	2.12±0.68	2.41±0.65^{e}	2.16±0.56^{a}	2.02±0.57^{ab}	1.20±0.29*
D(10)	2.16±0.52	2.11±0.47	2.11±0.77^{a}	2.02±0.59^{ab}	1.33±0.39^{*a}
E(10)	2.42±0.48	1.76±0.69^{*e}	2.04±0.66^{a}	1.91±0.63^{*ab}	1.33±0.35^{*a}
F(10)	1.94±0.38	1.92±0.85	2.16±0.92^{a}	1.68±0.59^{a}	1.09±0.39*

Note: According to the post-analysis of Kruskal-wallis test, statistical difference compared with the group at 0h is marked as *; Compared with group A, the marker with statistical significance is marked as [a]; Compared with group B in the same period, the marker with statistical significance is marked as [b]; Compared with group C, the marker with statistical significance is marked as [c]; Compared with group D, the marker with statistical significance is marked as [d]; Compared with group E in the same period, the marker with statistical significance is marked as [e]; Compared with group F at the same time, the mark with statistical significance is marked as [f].

Table II. Comparison of FIB content between different groups (mg/200ml, x ± s).

Groups (n)	0h	3h	5h	7h	9h
A (10)	245.56±25.92^{bcd}	219.09±20.20*	220.93±18.56^{bcdef}	214.73±16.40^{*cdef}	205.04±16.90^{*ed}
B (10)	222.88±24.66^{a}	215.89±25.11	213.32±15.12^{a}	204.67±15.33^{acde}	207.18±15.23
C (10)	225.80±23.15^{a}	223.88±17.24	198.90±12.30^{a}	199.69±26.48^{ab}	195.16±13.98
D (10)	224.51±19.69^{a}	210.51±27.01	207.56±18.18^{a}	209.69±12.52^{ab}	195.32±16.50^{*a}
E (10)	240.04±16.78	233.87±21.17	205.12±16.98^{*a}	207.09±17.71^{*ab}	199.59±17.62^{*a}
F (10)	227.43±24.86	223.58±23.65	198.88±25.31^{*a}	200.99±20.08^{*a}	205.47±10.70*

Note: According to the post-analysis of Kruskal-wallis test, statistical difference compared with the group at 0h is marked as *; Compared with group A, the marker with statistical significance is marked as [a]; Compared with group B in the same period, the marker with statistical significance is marked as [b]; Compared with group C, the marker with statistical significance is marked as [c]; Compared with group D, the marker with statistical significance is marked as [d]; Compared with group E in the same period, the marker with statistical significance is marked as [e]; Compared with group F at the same time, the mark with statistical significance is marked as [f].

Table III. Test results of inter-subject effect of FvIII content.

Source	Dependent variable: FvIII.					
	Class III sum of squares	Free degree	Mean square	F	Signifi-cance	Partial Eta square
Revised model	63.100^{a}	29	2.176	6.453	0.000	0.409
Intercept	1004.597	1	1004.597	2979.366	0.000	0.917
Quick freezing method	1.268	1	1.268	3.759	0.054	0.014
Storage temperature	2.556	2	1.278	3.790	0.024	0.027
Time	41.141	4	10.285	30.503	0.000	0.311
Storage temperature of quick-freezing method	6.059	2	3.030	8.985	0.000	0.062
Quick freezing method × time	3.351	4	0.838	2.484	0.044	0.035
Storage temperature × time	4.653	8	0.582	1.725	0.093	0.049
Quick freezing method × storage temperature × time	4.072	8	0.509	1.510	0.154	0.043
Error	91.040	270	0.337			
Total	1158.737	300				
Corrected total	154.140	299				
A. R square=0.409 (adjusted R square=0.346)						

FvIII content factor analysis of variance results

The content of FvIII is taken as the dependent variable. The quick-freezing method, storage temperature and time are set as fixed factors for one-way analysis of variance. The results shows that there is a significant effect of storage temperature, a significant interaction between quick-freezing method and storage temperature, and a significant interaction between quick-freezing method and time. There is a significant effect of time (P<0.05) (Table III).

FIB content factorial analysis of variance results

The FIB content is taken as the dependent variable. Quick freezing method, storage temperature and time are set as fixed factors. The analysis shows that there are significant effects of quick-freezing method and time, and there is significant interaction between quick-freezing method and storage temperature (P<0.05) (Table III).

DISCUSSION

Cryoprecipitation is applicable to infectious and hemorrhagic diseases, and can effectively reduce the amount of blood transfusion and the amount of antibiotics used (Li *et al*., 2014). Fresh frozen plasma contains abundant coagulation factors. With the increase of clinical use of coagulation factors, stricter preparation standards provide guarantee for the preparation quality of cryoprecipitates, especially for the protection of the rich coagulation factors (May *et al*., 2021; He, 2022).

Table IV. Inter-subject effect test results of FIB content.

Source	Dependent variable: FIB					
	Class III sum of squares	Free degree	Mean square	F	Signifi-cance	Partial Eta square
Revised model	49538.201[a]	29	1708.214	4.399	0.000	0.321
Intercept	13578177.311	1	13578177.311	34970.271	0.000	0.992
Quick freezing method	5432.444	1	5432.444	13.991	0.000	0.049
Storage temperature	468.683	2	234.342	0.604	0.548	0.004
Time	29809.586	4	7452.397	19.193	0.000	0.221
Storage temperature of quick-freezing method	2409.840	2	1204.920	3.103	0.047	0.022
Quick freezing method × time	1839.732	4	459.933	1.185	0.318	0.017
Storage temperature × time	4161.692	8	520.212	1.340	0.224	0.038
Quick freezing method × storage temperature × time	5416.224	8	677.028	1.744	0.088	0.049
Error	104834.986	270	388.278			
Total	13732550.499	300				
Corrected total	154373.187	299				
A. R square=0.328 (adjusted R square=0.255)						

The preparation process of cold precipitation is affected by many factors, especially the preparation method and preservation environment. Wei *et al*. (2023) found that fresh frozen plasma and cryoprecipitate would be affected by preparation time and temperature, resulting in changes in coagulation factor content. The American Association for the Development of Blood and Biological Agents (AABB) and the food and drug administration (FDA) once pointed out that the preparation process of cryoprecipitate needs to be completed within 8 h. At the same time, it was also emphasized that the 20-24 h of O-type frozen whole blood can be cryoprecipitate processed, which still can obtain a variety of coagulation factors (Obaid *et al*., 2022). Therefore, this study takes the preparation method and storage temperature as the main parameters to observe the changes of FvIII and FIB content in the cryoprecipitate precipitation. At the same time, only type O blood samples are collected to reduce the result deviation of the included samples affected by blood type differences.

Feedback activation of factor XI by thrombin has been shown to play a critical role during the amplification phase of thrombin generation, contributing to thrombus formation and hemostasis (Rühl *et al*., 2022). The imbalance between the procoagulant and anticoagulant systems is one of the stress responses of the body to sepsis infections, and adjusting the FvIII levels in transfused plasma can optimize the balance between procoagulant and anticoagulant factors (Stahl *et al*., 2020). Treatment based on FVIII replacement therapy can lead to inhibitor development that renders FVIII concentrate infusions ineffective (Cadé *et al*., 2022). From the research results, the highest FvIII level appeared in group B at 5h, while the lowest appeared in group A at 9h. The trend in each group was a transient increase followed by a slow decrease, B group reached a peak at 5h, and all groups were able to meet the qualified standard. The highest FIB level appeared in group E at 0h, while the lowest appeared in group C at 9h. The trend in each group was a slow decrease, with group A showing a slower decrease. All groups obtained qualified cold precipitate FIB. Currently, there is little research on the rapid freezing time during the preparation of cold precipitates. The Technical Operational Procedure for Blood Stations (2019 edition) (Song, 2019) recommends completing cold precipitate preparation within one hour, using rapid freezing at -30°C. However, the time should not exceed 1 hour, as prolonged time may affect cold precipitate quality. However, from the results of this study, even in cases where the rapid freezing time was 120 min, no severe adverse effects were observed on the quality of the cold precipitate. Therefore, it can be inferred that in practical work, factors that extend the rapid freezing time of the cold precipitate due to different reasons will not significantly affect the final preservation outcome. It has been suggested that under room temperature (22-24°C) conditions, cold precipitates offer great advantages as there is a higher risk of bacterial growth and increased platelet reactivity. At lower temperatures (2-6°C), the activity of multiple coagulation factors is decreased in the cold precipitate, although the degree of decline is less than that under room temperature conditions (Kobsar *et al*., 2022).

In this study, it was found that a rapid freezing time of 30 min using a platelet freezer and storage at -4℃ resulted in higher levels of FvIII and a more stable relative content within 0-9h. It has also been reported that normal use of clotting factors is safe within 24 h after thawing. Within the subsequent 5 days (120h after thawing), qualified FVIII concentrates or antihemophilic cold precipitates can still be obtained (Bahreini *et al*., 2021). Thomson *et al*. (2021) found that under different storage conditions, fibrinogen is not significantly affected, but coagulation factors within cold precipitates decreased significantly after 120h. However, potential bacterial contamination risks can be effectively avoided under temperatures of 2-6°C. This finding is similar to the results of this study and indicates that storage at 4℃ is more beneficial for increasing coagulation factor levels.

The further analysis found that the content of FvIII would be significantly affected by the storage temperature and time. There is a significant interaction between quick-freezing method and storage temperature. There was significant interaction between quick-freezing method and time ($P<0.05$). FvIII, as an important coagulation factor in cryoprecipitation, is an important factor in the treatment of hemorrhage. According to thrombin determination, thrombus kinetic diagram and other tests, the factor can be used for up to 14 days when stored at 2-6 ℃. Long-term storage period, liquid state and MB treatment improve its usability for bleeding treatment and provide a safe pathogen ingredient (Rapaille *et al*., 2021). Therefore, in order to maintain a relatively high level of FvIII content in the precipitation, a relatively high storage temperature, such as 2~6 ℃ (4 ℃ in this paper), can be selected under the condition of equal storage time. Moreover, the quick-freezing method changes with time. The quick-freezing temperature is a non-independent effect factor, which should be comprehensively considered in combination with the storage temperature and storage time. The FIB content was found to be influenced by the rapid freezing method, time, and interaction effects between rapid freezing method and storage temperature ($P<0.05$). Therefore, it can be inferred that the rapid freezing method is the dominant factor affecting FIB content under the same storage time. Based on the results of this study, group A exhibited the highest FIB content after completing plasma rapid freezing. In light of the important hemostatic effect and effective anticoagulant activity of FIB, a preparation plan that can easily obtain higher content can be selected based on storage time and rapid freezing method (Napodano *et al*., 2021). Furthermore, it was observed that FIB content gradually decreased with time. It can be inferred that exposing thawed plasma to a certain temperature for up to 60 min is relatively safe. The preparation process of cold precipitates has been effectively improved by the application of electronic information management, which has enhanced work efficiency (Raycraft *et al*., 2022).

In summary, different rapid freezing methods and storage temperatures have an impact on the FvIII and FIB content in the cold precipitates of fresh plasma. The optimal rapid freezing method was determined to be a platelet freezer for 30 min at a storage temperature of -4℃, which resulted in a slower decline in the coagulation factor content in the cold precipitates. This study did not compare the coagulation factor content under different time, storage conditions, and different preparation instruments. In the future, efforts can be made to improve coagulation factor content or reduce the content of coagulation factors in blood products by not being limited to environments with the same temperature that are unchanged for a long time. Additionally, this study did not analyze the differences in the impact of various factors, such as rapid freezing methods and storage temperatures, on non-O blood type samples. Therefore, future studies will further analyze such differences.

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IRB approval

This research was carried out with the approval of Research Guidance Workshop Committee (Changsha Blood Center and Xiangtan Blood Station).

Ethical statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Statement of conflict of interest

The authors have declared no conflict of interest.

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