

Comparative Study of Autologous Fibrin Glues Prepared by Cryo-Centrifugation, Cryo-Filtration, and Ethanol Precipitation Methods

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Received May 26, 1999; accepted August 3, 1999

To establish a speedy preparation method for the fibrinogen-rich fraction (FRF) from autologous plasma using fibrin glue, we compared the concentrations and yields of coagulation factors in FRF prepared by 3 methods. Human plasma from healthy volunteers was divided into 3 samples. Two samples were frozen at -20°C in a freezer and defrosted in a 4°C water bath. One sample of defrosted plasma was centrifuged and FRF was obtained (C method). Another sample of defrosted plasma was filtered and FRF was obtained (F method). The last sample was treated with cold ethanol(1/10) in a 4°C water bath and FRF was obtained after centrifugation (E method). The concentrations of fibrinogen, fibronectin, factor XIII, and plasminogen in each obtained FRF were measured and yields were calculated. (1) The volume of FRF obtained by the E method was greater than that by the C method, but less than that by the F method. While the variation in volume obtained by the E method was the lowest among the 3 methods; (2) the concentrations of fibrinogen obtained by the E and C method were similar, but the yield from the E method was the highest; (3) the concentration and yield of fibronectin from the E and C method were similar and were greater than those by the F method; (4) the concentration and yield of factor XIII from the E method were significantly higher than those from the other methods; (5) the E method preparation time was about 1 h, the shortest among the 3 methods. These results indicate that high quality FRF from autologous plasma can be prepared easily and within 1 h by the E method.

Key words fibrin glue; fibrinogen; ethanol; autologous plasma; cryoprecipitate; rapid preparation

Fibrin glue consists of fibrinogen, coagulation factor XIII (F-XIII), thrombin, CaCl_2 , and aprotinin as inhibitors of the fibrinolytic system.¹⁾ Fibrin glue reacts according to the physiological hemostatic mechanism and coagulates and adheres bleeding tissues.²⁾ Fibrin glue products are commercially available and are widely used as biological adhesives in surgery.^{3–5)} However, fibrin glues are produced from human plasma collected from healthy volunteers, and are heated at 60°C for more than 10 h to inactivate hepatitis B virus and human immunodeficiency virus-1.⁶⁾ Patients in whom the fibrin glue is used are thus placed at risk of infection by thermostable pathogens such as Parvovirus B19 and Prions.^{7,8)} In addition, since each constituent of fibrin glue is classified into several subtypes,^{9,10)} its use is associated with a risk of allergic reaction. For the prevention of these adverse effects, fibrinogen-rich fraction (FRF) produced from a patient's autologous plasma is recommended for use as the fibrin glue.^{11,12)} However, it takes about 2 d to obtain FRF by the currently used preparation method, and thus autologous FRF cannot be prepared quickly enough for emergency patients urgently needing surgery.

The goal of our study is to develop an automatic device to prepare high quality FRF rapidly and easily using a patient's own plasma. In the present study, to establish the optimal preparation method for obtaining FRF from a patient's own plasma, we compared the concentrations and yields of coagulation factors (CFs) in FRF prepared by 3 methods, a cryo-centrifugation (C method), a cryo-filtration (F method), and an ethanol precipitation (E method).

MATERIALS AND METHODS

Materials Human plasma from healthy volunteers, excluded by the alanine aminotransferase (ALT) test, was obtained from the Yamaguchi Red Cross Blood Center (Yama-

guchi, Japan). The plasma was divided into 3 disposable and sterilized bags (T015CJ, Terumo Co., Tokyo, Japan), and used. The assay kits used for fibrinogen, plasminogen, fibronectin, F-XIII and fibrin degradation products (FDP) were ILtest PT-Fibrinogen (Coulter Co., Tokyo), ILtest Plasminogen (Coulter), Fibronectin (Opsonic Protein) (Boehringer Mannheim Co., Tokyo), Latex Kit F-XIII (Hoechst Japan, Tokyo), and FDP reagent (ND) (International Reagent Co., Kobe, Japan), respectively. All chemicals used were obtained from commercial sources and were of analytical reagent grade.

Preparation of FRF The divided plasma was treated under various conditions, as outlined below. Two bags of the divided plasma were horizontally shaken during the freezing periods at 70 cycles/min for about 1 h and were completely frozen. The bags were then horizontally shaken during the defrosting periods at 70 cycles/min in a 4°C water bath for about 1.5 h. One defrosted bag was centrifuged at 3000 rpm for 15 min at $0-4^{\circ}\text{C}$, and the supernatant was removed. The precipitate was collected as FRF by the C method.¹³⁾ Defrosted plasma in another bag was filtered using a hollow fiber membrane filter (Asahi BCC-1-C, Asahi Medical Co., Tokyo, Japan) in a 4°C water bath *via* a peristaltic pump. Then the filter was warmed in a 37°C water bath, and the gel was removed from the hollow fiber due to the counter-revolution of the peristaltic pump. The gel was collected as FRF by the F method. The last batch of plasma was thoroughly mixed with cold ethanol (one tenth volume), and was incubated for 30 min in a 4°C water bath. The sample was then centrifuged at 3000 rpm for 15 min at $0-4^{\circ}\text{C}$, and the supernatant was removed. The precipitate was collected as FRF by the E method.¹⁴⁾

Lysing Test of Fibrin Glue FRF prepared by the 3 methods was mixed with aprotinin, and then a mixture of thrombin (100 units/ml) and CaCl_2 (0.2%) was added while

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agitating the solution. This glue was incubated in phosphate buffered saline (PBS) containing plasminogen (200 μ g/ml) and urokinase (200 units/ml) at 37 °C, and the medium was sampled several times.

Measurement of Adhesive Strength Adhesive strength of FRF was measured as follows: Two ground glasses of 4 cm², bound on cubic wood with chemical adhesive, were prepared. Ten microliters of FRF was applied on a glass and 10 μ l of the mixture of thrombin (100 units/ml) and CaCl₂ (0.2%) was applied on another glass. Both applied surfaces were adhered together and were pressed at 50 g/cm² for 30 s. After 10 min, the glasses adhered through FRF were fixed in a tension measuring device (SV-55, Imada Manufact. Co., Ltd., Toyohashi) and maximum tensile adhesive strength was measured.¹⁵⁾

Analytical Methods The concentrations of fibrinogen and plasminogen in each plasma sample and FRF were determined using commercial kits supplied with the automated coagulation analyzer-ACL3000 (Coulter Co., Tokyo). Fibronectin was determined by the immunoprecipitation method. F-XIII and FDP were determined by the latex agglutination method.

Calculation of Percent Yield % yield of CFs after treatment was calculated as follows; % yield=(concentration of CF in the FRF) \times (volume of the FRF)/((concentration of CF in raw plasma) \times (volume of raw plasma)) \times 100

Statistical Analysis Each value was expressed as mean \pm S.D. of 9 experiments. Comparisons among the three methods were made using Student's unpaired *t*-test and one-way analysis of variance (ANOVA) followed by Scheffe's test with StatView J4.02 for Macintosh, and differences were considered to be statistically significant when *p*<0.05.

RESULTS

Collection Volume The mean volume of FRF by the C, F, and E methods was 3.4 \pm 0.8, 7.8 \pm 1.6, and 5.3 \pm 0.8 ml per 100 ml of raw plasma, respectively. The volume of FRF from the E method was greater than from the C method but less than from the F method. The variation of volume by the E method was the lowest among the 3 methods.

Fibrinogen The fibrinogen concentration in raw plasma was 2.5 \pm 0.5 mg/ml. The concentrations and yields of fibrinogen in FRF obtained by each method are shown in Fig. 1. While the concentration of fibrinogen from the E method was similar to that obtained by the C method, the yield was significantly higher than from the C or F methods.

Fibronectin The fibronectin concentration in raw plasma was 0.45 \pm 0.08 mg/ml. As shown in Fig. 2, the concentrations and yields of fibronectin from the E and C methods were similar and significantly higher than those from the F method.

F-XIII The fibronectin concentration in raw plasma was 1.8 \pm 0.5 units/ml. The concentration and yield of F-XIII in FRF from the E method were significantly higher than those from the C or F methods (Fig. 3).

Plasminogen The plasminogen concentration in raw plasma was 1.01 \pm 0.06 units/ml. The concentration of plasminogen in FRF by the F method was significantly lower than those by the C or E methods, but the yield by the C method was the lowest (Fig. 4).

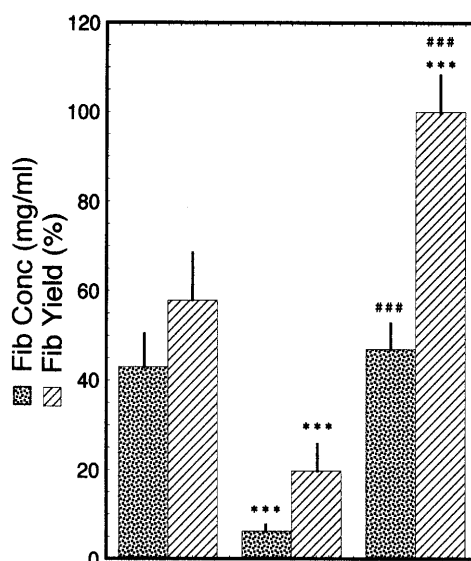


Fig. 1. Comparison of the Concentration and Yield of Fibrinogen among the FRFs Prepared by Three Methods

***: *p*<0.001 (vs. C method), ###: *p*<0.001 (vs. F method).

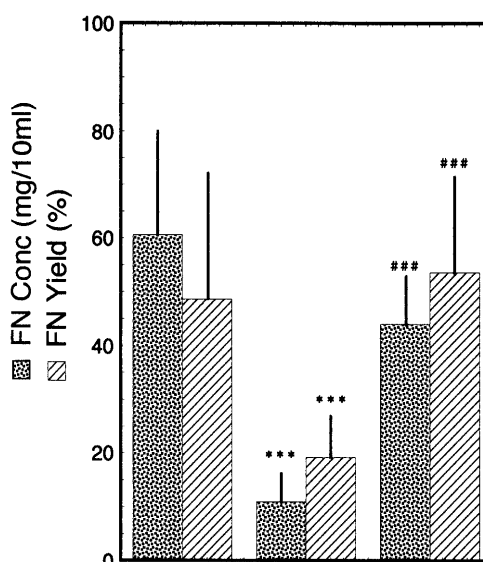


Fig. 2. Comparison of the Concentration and Yield of Fibronectin among the FRFs Prepared by Three Methods

***: *p*<0.001 (vs. C method), ###: *p*<0.001 (vs. F method).

Fibrinolysis The time course of FDP in the medium is shown in Fig. 5. Each fibrin clot of FRF, prepared by the 3 methods, was slightly degraded with the passage of time and generated a small amount of FDP. FDP generation was not significantly different among FRF generated by the 3 methods during 8 h, but FDP generated by the F method was significantly higher than that by C and E methods after 24 h.

Adhesive Strength The adhesive strength with raw plasma was 0.12 \pm 0.02 kg/cm². The strength with FRF by the C, F, and E method was 1.17 \pm 0.30, 0.25 \pm 0.08, and 1.15 \pm 0.21 kg/cm², respectively.

Preparation Time The total preparation time for FRF by the 3 methods was compared. As shown in Table 1, the E method required the shortest amount of time at about 1 h.

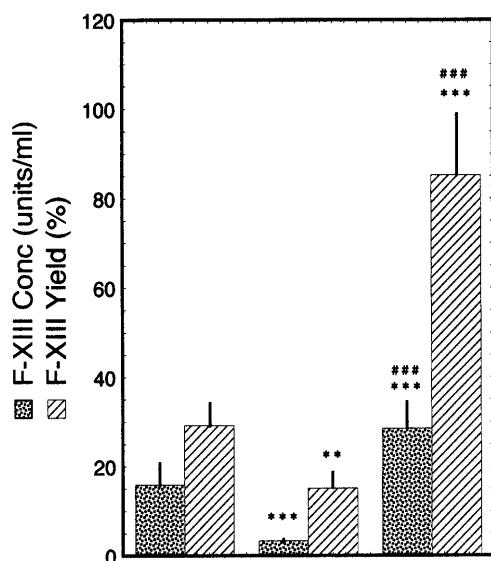


Fig. 3. Comparison of the Concentration and Yield of F-XIII among the FRFs Prepared by Three Methods

: $p < 0.01$, *: $p < 0.001$ (vs. C method), ###: $p < 0.001$ (vs. F method).

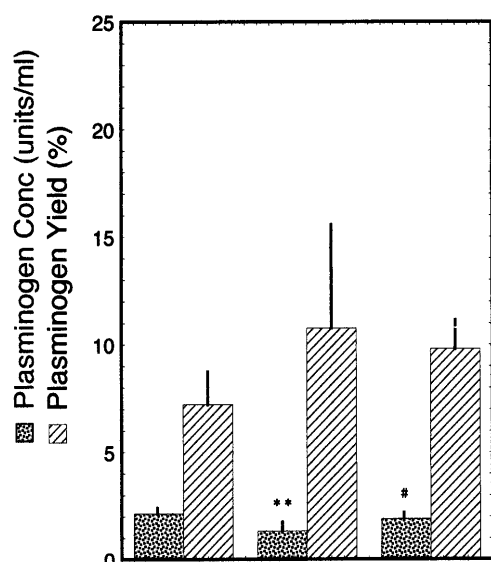


Fig. 4. Comparison of the Concentration and Yield of Plasminogen among the FRFs Prepared by Three Methods

**: $p < 0.01$ (vs. C method), #: $p < 0.05$ (vs. F method).

DISCUSSION

Autologous FRF, which is a widely used fibrin glue, can be prepared by different methods.^{14,16–19} However, it takes about 2 d to obtain FRF by the general preparation method (cryoprecipitate centrifugation method), and thus autologous FRF cannot be prepared quickly enough for emergency patients urgently needing surgery. In our previous paper,¹³ we established a preparation method by which FRF containing high concentrations of coagulation factors could be prepared within 4 h (C method). However, this method involves various processes to obtain the FRF; plasma collection, freezing, shaking, defrosting at low temperature, precipitate collection, *etc.* About 70% of the total preparation time is occupied by the freezing and defrosting processes. These processes inhibit a quicker preparation of FRF and required a large

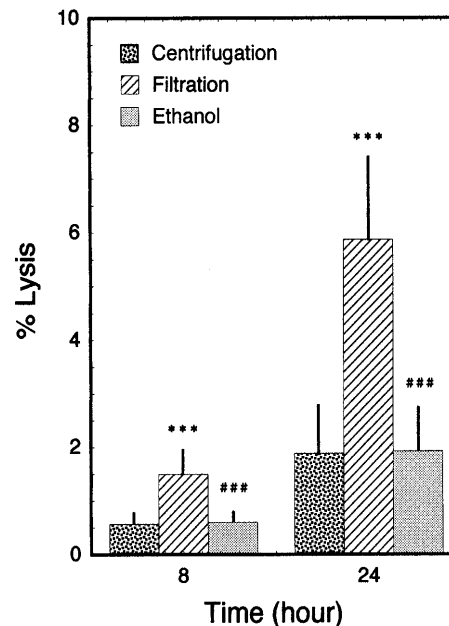


Fig. 5. Comparison of Lysing Property among the Fibrin Glues of FRFs Prepared by Three Methods

***: $p < 0.001$ (vs. C method), ###: $p < 0.001$ (vs. F method).

Table 1. Comparison of Properties among the Preparation Methods

Item	C method	F method	E method
Adhesive strength (kg/cm ²)	1.15 ± 0.33	0.23 ± 0.09***	1.18 ± 0.28
Preparation time (h)	3.02 ± 0.10	3.04 ± 0.09	0.98 ± 0.09***
Handling	Normal	Difficult	Easy
Quantative reduction	Normal	Difficult	Easy
Qualitative equality	Low	Low	High

***: $p < 0.001$ (vs. C method).

amount of electric power. Moreover, a centrifuge is usually used for the collection of a patient's plasma and precipitate by the C method, and centrifugation impedes development of a small preparation device. The F method does not require a centrifuge and the E method does not require freezing and defrosting. The purpose of our study is to develop an automatic device to prepare high quality FRF rapidly and easily using a patient's own plasma. In this paper, to establish a speedy preparation method for FRF, we compared the concentrations and yields of CFs in FRFs prepared by these 3 novel methods. Our results indicate that high quality FRF from a patient's own plasma could be prepared easily and quickly by the E method. Moreover, we recognized that quantitative reduction of raw plasma by the E method was easier than by the other methods, and that variations in the quality of FRF obtained by the E method were the smallest.

Our study shows that a fibrinogen concentrate suitable for fibrin glue can be obtained by the E method from a patient's own plasma. Generally, the properties of fibrin glue are determined mainly by the presence of fibrinogen, F-XIII and fibronectin.^{20–22} Fibrinogen content is related to the adhesive strength of the fibrin glue, which is increased by F-XIII-mediated cross-linking of fibrinogen. The quantity of fibrinogen and F-XIII recovered by the E method was approximately 100% and was significantly higher than that by the C or F methods. Moreover, the concentrations of plasminogen, which is the precursor of fibrinolytic enzyme, in FRFs ob-

tained by the E and C methods, were slightly higher than that from the F method but fibrinolysis was not accelerated. These results indicate that the E method is superior than the other methods regarding the CFs contents in FRF.

The FRF obtained by the E method had the same adhesive strength as the C method. Casali B *et al.* reported that the adhesive strength of fibrin glue containing 78 mg/ml of fibrinogen was 117 g/cm².¹¹⁾ The discrepancies in measured adhesive strength may be due to different methods, since they used the Gottlob mouse skin method and we used the ground glass method. Namely, we measured adhesive strength in the vertical direction to the adhesive surface, whereas they probably measured strength in the horizontal direction.

In emergency surgery, the speed of the E method may be advantageous. Further the cost of FRF obtained by the E method is mainly from disposable vessels and ethanol and compares favorably with that of commercial preparations.

In conclusion, our study shows the E method can prepare easily and quickly high quality FRF from a patient's autologous plasma within 1 h.

Acknowledgement We gratefully acknowledge the Yamaguchi Red Cross Blood Center for donating human plasma from healthy volunteers that had been excluded by the ALT test.

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