IV. DISCUSSION

Because of the high risk of viral transmission after infusion of the pooled fibrinogen concentrates, as they were then manufactured, their used in the United States was prohibited by the Food and Drug Administration in 1978. Subsequently, procedures using autologous or single donor component for the production of fibrin glue were developed in both the United States and other countries.

In this study, fibrinogen solutions were prepared by several methods, which based on precipitation. All of these methods could precipitate several proteins in plasma including fibrinogen. The precipitate from these methods could dissolve in the supernate that leaving for reconstitution except both polyethylene glycol precipitation methods. Polyethylene glycol is a nonionic and water-soluble polymer that used for fractional precipitation of protein. The viscous of solution is increase when its sized is increase (Ingham, 1990). The fibrinogen is a large molecule, so the polyethylene glycol precipitation must be used a high polymer. Centrifugation was not a suitable method for fibrinogen concentration by polyethylene glycol precipitation because the precipitate was become sticky. Its sticky may be resulted from the viscosity of high polymer and high protein concentration. It would be removed the polyethylene glycol by the ion-exchange chromatography, affinity chromatography, ultrafiltration and salt-induced phase separation which were use the expensive instruments and time-consuming. The precipitate from both 10% ethanol precipitation methods was not dissolved in 37 $^{\circ}$C waterbath. It may be resulted from the exposure time and temperature to organic solvent. In order to precipitate protein by organic solvent such as ethanol, the precipitating procedure is normally used near 0 $^{\circ}$C or less because organic solvents tend to denature proteins at higher temperature. But the precipitates from both absolute ethanol precipitation methods were dissolved. It might be the result

from the exposure time and temperature of plasma with absolute ethanol in this method was shorter and lower than both 10% ethanol precipitation methods.

All of these fibrinogen precipitation methods can precipitate but the abilities of each method in difference types of protein were differed. Cryoprecipitation is a simple method of fractionation derives from the observation that when frozen plasma is allowed to thaw at a low temperature (0-8 $^{\circ}$C), a small amount of gelatinous material amounting to about 3% of the plasma protein remains undissolved. It was known from the report of Ware, Guest and Seegers in 1947 that this material contains a high proportion of the fibrinogen of the original plasma (Bidwell E, 1974). Cryoprecipitate also contains factor VIII, Factor XIII, von Willebrand's factor (vWF), and other cryoproteins. Cryoprecipitation is a freeze-thaw cycle so fibrinogen concentration is less than repeat cryoprecipitation, which are two freeze-thaw cycles. Saturated ammonium sulfate precipitation is known as salting-out. It is a result of the competition between the added salt ions; ammonium sulfate [$(NH_4)_2SO_4$] and the other dissolved solutes for molecules of solvation (Voet D and Voet JG, 1995). The dissolved solute in this situation is meant to fibrinogen. At high salt concentration [saturated $(NH_4)_2SO_4$], many of added ions are solvated the amount of bulk available becomes insufficient to dissolve other solutes including fibrinogen. A higher concentration of fibrinogen from saturated ammonium sulfate precipitation and followed by cryoprecipitation than that from saturated ammonium sulfate precipitation is resulted from the composition of two precipitation methods. Cryoprecipitation in the second step of saturated ammonium sulfate precipitation and followed by cryoprecipitation was precipitated the remaining fibrinogen that dissolved in the solution mixture. Ethanol is organic solvent. It is miscible with water, but yield a significant heat of solution. It has tendency to denature proteins, especially at temperature above 0 $^{\circ}$C (Englard S and Seifter S, 1990). In this study, absolute ethanol

precipitation was performed in icebath during the time of adding and stood the mixture at 0 °C for 30 minutes before centrifugation. So denaturation of fibrinogen and other proteins were not occurred. As the same as absolute ethanol precipitation, the further step of absolute ethanol precipitation and followed by cryoprecipitation was frozen the mixture at –20 °C, so denaturation of fibrinogen and other proteins were not occurred. Fibrinogen concentration from absolute ethanol precipitation and followed by cryoprecipitation was lower than that from absolute ethanol precipitation in this study. This was differed from repeat cryoprecipitation and saturated ammonium sulfate It may be results from the number of samples in absolute ethanol precipitation and followed by cryoprecipitation was less than the others. All of these precipitation methods contained several proteins, especially albumin, it does not mean that fibrin glue from these fibrinogen solutions is inadequate in clinical application, because additional proteins precipitated in them do not influence fibrin clot formation or its stability. However, too much albumin may interfere with fibrin gelation by increasing fibrin solubility, and its biological properties may modified (Park MS, *et al.* 1993)

Concentration of fibrinogen determined by Ratnoff's method was usually higher than modified thrombin time method. This may resulted from Ratnoff's method is determined its tyrosine-liked activity, while modified thrombin time is determined only clottable fibrinogen. Other proteins contain tyrosine in their molecules. They may trapped in fibrin clot and could react with Folin-Ciocalteu Phenol reagent in Ratnoff's method. Concentration of fibrinogen from saturated ammonium sulfate precipitation, and saturated ammonium sulfate precipitation and followed by cryoprecipitation determined by modified thrombin time method were higher than that determined by Ratnoff's method. These may result from several reasons. Firstly, ammonium sulfate molecules that remains in fibrinogen solution, which traps in fibrin clot, may interfering the reaction of Folin-Ciocalteu Phenol reagent and tyrosine in this fibrin

clot. Secondly, fibrin from both methods of saturated ammonium sulfate precipitation may be soluble fibrin. It would be loss or partially dissolved while it was washed 3 times by 0.85% NaCl. All of these precipitation methods could precipitate fibrinogen without damages its molecule. Thrombin time of fibrinogen solution from saturated ammonium sulfate precipitation and followed by cryopercipitation was not correlated to its fibrinogen concentration. This may resulted from thrombin concentration. In thrombin time, 5 NIH units/ml thrombin was used, while 75 NIH units/ml thrombin was used in modified thrombin time. It was 15 times lower. So the lower thrombin concentration, the slower rate for fibrin formation.

In urea solubility test, the screening test for factor XIII deficiency, it was found that fibrin clot after adding 5 NIH units/ml thrombin to fibrinogen solution from both saturated ammonium sulfate precipitation methods and both absolute ethanol precipitation methods were completely dissolved within 24 hours after adding 5 M urea solution while as that from cryoprecipitation and repeat cryoprecipitation were not dissolved. This may be resulted from ammonium sulfate and ethanol molecules that remain in fibrinogen solution. Firstly, they may interfere the transamidase activity of factor XIII that catalyze the covalent cross-link between glutamine and lysine side chain. Secondly, they may be covered around the glutamine and lysine side chain, result in that enzyme could not react with the glutamine and lysine side chain. In the method that used $CaCl_2$ to activate prothrombin to thrombin, and thrombin would convert fibrinogen to fibrin, fibrinogen solution from both methods of saturated ammonium sulfate precipitation were not formed fibrin clot after adding $CaCl_2$ but these fibrinogen solutions were formed fibrin clot after adding 5 NIH units/ml. This may be resulted from these fibrinogen solution did not contain prothrombin.

From these results, the best method for fibrinogen preparation in this study was repeat cryoprecipitation because it was provided a high concentration and high quality

of fibrinogen, less interference, simple and it was also contained factor XIII that be sufficient for stabilize fibrin clot. The second choice was cryoprecipitation because it was provided a high concentration, but less than repeat cryoprecipitation, and high quality of fibrinogen and also contained factor XIII that be sufficient for stabilize fibrin clot.

In this study, thrombin preparation method was based on *pI* to precipitate the coagulation protein in the first step. The second step was added $CaCl_2$ to generate Ca^{2+} to activate prothrombin to thrombin. The solution mixture was added the precipitating agents after removing the fibrin clot to reprecipitate thrombin in the solution mixture. Thrombin solution from acetone, absolute ethanol and polyethylene glycol precipitation had thrombin-liked activity, which could convert fibrinogen to fibrin, while as that from saturated ammonium sulfate precipitation had not. Thrombin solution from saturated ammonium sulfate precipitation could not convert fibrinogen to fibrin. The result of thrombin preparation by saturated ammonium sulfate precipitation was correlated to the result from factor XIII screening test that used 0.025 M $CaCl_2$ added into fibrinogen solution from saturated ammonium sulfate precipitation. This was suspected that no thrombin in this solution. This may be the result from that saturated ammonium sulfate could not precipitate prothrombin and thrombin in fibrinogen and thrombin solution from saturated ammonium sulfate precipitation, respectively. Activity of thrombin from absolute ethanol precipitation determined by thrombin time method was not differed from acetone precipitation while as that from polyethylene glycol precipitation was lower. From these results, absolute ethanol precipitation is the superior method for thrombin preparation. Thrombin solution from this present study had an immediately advantage in *in vitro* for laboratory use. It was used for thrombin time test in Department of Clinical Microscopy, Faculty of Associated Medical Science, Chiang Mai University.

Commercial thrombin (5 NIH units/ml, Sigma Co., Cat. No T-9135) was substituted by homemade thrombin solution. A price of this commercial thrombin was 38.40 bahts/unit. Thrombin-liked activity of our preparation was about 40 NIH units/ml. One unit of 450 ml CPD-blood, which provides 200 ml of platelet poor plasma, was provided 50 ml of thrombin solution. It was also provided thrombin-like activity 2000 NIH units. Its cost was about 0.50 baht/unit. It could be decrease the cost of this test about 77 times. It was suspected that this thrombin-liked activity solution was contained thrombin or only thrombin-liked activity substance. This should be identified by amino acid sequencing or specific substrate assay (Mann KG, *et al.* 1987)

To define an appropriate ratio of fibrinogen solution, which contains antifibrinolytic agent and antibiotic, and thrombin solution, which contains $CaCl_2$ for fibrin glue preparation. In wound healing process, the substrate phase is maintained for about 5 days when collagen fibers begin to grow. Therefore, it is not desirable that the fibrin clot survives too long because it may interfere with the normal process of wound healing (Seidentop KH, 1986). EACA was used as antifibrinolytic agent in this study because of its cheaper and easier to obtain than aprotinin, a strongest fibrinolytic inhibitor (Park MS, 1993). EACA is slightly less effective than aprotinin (Seidentop KH, 1986). EACA at concentration of more than 10 mg/ml, have also been used, mostly for "homemade" products (Rodosevich M, 1997). EACA concentration of 30 mg/ml has adequate inhibitory effects to prevent autofibrinolysis of autologous fibrin glue (Park MS, 1993). The least concentration of EACA that could prevent autofibrinolysis in this study was 7.5 mg/ml. However, it could stabilize fibrin glue more than 7 days. The ratios of fibrinogen and thrombin solution were varying to define an appropriate ratio for fibrin glue. It is well known that the adhesive strength of fibrin glue is directly proportional to the concentration of fibrinogen (Seidentop KH, 1998). Final concentration of fibrinogen in the 1:1 mixture before it forms fibrin clot was

lower than the 2:1 mixture in this study. Therefore, it was provided a low adhesive strength of fibrin glue. Elasticity of fibrin glue depends on the degree of covalent cross-linking which lead to the formation of α-chain polymers. This important calcium-dependent cross-linking reaction which participates in the formation of the clot is highly dependent of factor XIII. Elasticity is particularly important when fibrin glue is applied on cut surfaces and must withstand body fluid pressure (Bounouf-Rodosevich M, *et al.*, 1990).

The stability and elasticity of fibrin glue in this present study compared with fibrin glue prepared by Thai Red Cross Society were not differed. Of their stability, the different types of antifibrinolytic agent were compared. It was represented that 7.5 mg/ml EACA in fibrin glue in this study was as effective as 12.5 mg/ml transamine in fibrin glue prepared by Thai Red Cross Society. Of their elasticity, it was represented that the degree of covalent cross-linking of fibrin glue prepared in this study was as effective as fibrin glue prepared by Thai Red Cross Society. However, the adhesive strength of them was differed. It was resulted from the final concentration of fibrinogen in the mixture before it forms fibrin clot as described above. Fibrin glue in this present study was composed of 2:1 ratio of fibrinogen and thrombin solution while as fibrin glue prepared by Thai Red Cross Society was composed of 1:1 ratio of fibrinogen and thrombin solution. Therefore, the final concentration of fibrinogen in fibrin glue from this present study was higher than that prepared by Thai Red Cross Society.

In conclusion, fibrinogen solution from repeat cryoprecipitation and cryoprecipitation in this present study was provided a high concentration and high quality of fibrinogen, less interference, unsophisticated and it was also contained factor XIII that sufficient for stabilize fibrin clot. Thrombin solution from absolute ethanol precipitation was provided desirable thrombin-liked activity and also simple. The

recommended ratio of fibrinogen and thrombin solution for fibrin glue was 2 parts of 7.5 mg/ml EACA and 2 mg/ml gentamicin in 1 ml of fibrinogen solution, and 1 part of 40 mmol/l $CaCl_2$ in 1 ml of thrombin solution. This was provided more than 7 days of stability, more than 5 mm of elasticity, and more than 200 g/cm^2 of adhesive strength. This novel method could be performed in routine hospital laboratory and low cost. This is a true single donor fibrin glue because both fibrinogen and thrombin solution were derived from 1 unit of CPD blood. It also decreases a risk of infection. These preparation methods must be performed in sterile condition and check its sterility before use. It should be more useful if its toxicity and clinical trial were studied. And thrombin preparation from cryoprecipitate-removed plasma should be further study for the highest benefit of donated CPD-blood.