Editor-in-Chief:
Dr. Nguyen Phuong Sinh

Received:
13/7/2023
Accepted:
20/8/2023
Published:
31/12/2023

Competing interests: The authors have no competing interests to declare.

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THE PROCEDURE OF MAKING FIBRIN GLUE BY FROZEN PLASMA METHOD ON EXPERIMENT

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ABSTRACT

Background: Currently, fibrin sealant (glue) is used in virtually every surgical specialty. The concentrated fibrinogen is produced by different methods as well as multiple sources of plasma. Objectives: Identified the process for making fibrin sealant (glue) using plasma frozen methods. **Methods:** Using rabbit's plasma to determine the optimal temperature and time to not only produce the highest fibrinogen concentration, but also achieve thrombin at the same time. **Results:** The average fibrinogen concentration yielding was similar at the time of freezing within 12h, 24h, and 48 hours. All were greater than fibrinogen concentration yielding of freezing within 2 hours. The average fibrinogen concentration yielding at temperatures of -18°C and -35°C was similar at the time of freezing of 12h, 24h, and 48 hours. However, this was lower at the time of freezing of 2h. The percentage of fibrinogen yielding when freezing within 12 to 48 hours at a temperature of -18°C is from 33.97 to 55.13%. The percentage of fibrinogen obtained when freezing within 12 to 48 hours at a temperature of -35°C is from 31.10 to 57.46%. **Conclusions:** Plasma was frozen at -18°C within 12 - 48 hours. Defrost and decant a portion of the plasma to collect fibrinogen. Creating thrombin by adding calcium chloride to the plasma. Mix fibrinogen with thrombin in a ratio of 1:1 to create fibrin glue.

Keywords: Fibrinogen; Fibrin glue; Thrombin

INTRODUCTION

Currently, fibrin glue is known and used more and more in virtually every surgical with the purpose of tissue adhesion, hemostasis, and wound healing. The main components of fibrin glue are fibrinogen and thrombin. Companies have extracted components of glue from multiple sources of plasma such as

cows, horses ... or plasma of many people to make fibrin glue. Their limitations are the risk of spreading pathogens through blood, complicated collection and high cost.

Therefore, the new trend that has been focused on by recent studies is the production of autologous fibrin glue. That is, the resulting glue product is used for the same object to reduce the risk of transmitting pathogens and to reduce the body's response to other glue of different species. Many studies showed that, each fibrinogen extraction method has its advantages and disadvantages. For example, the drug or chemical method will reduce the properties of the glue and the price is expensive. Some publications have shown that actual glue creation is not self-sufficient because the authors used only methods of extracting fibrinogen from their own blood, but using commercial thrombin to create glue[1]. In Vietnam, Vu Thi Kim Lien et al (2018) have successfully produced autologous fibrin glues with protamin[2]. Huynh Duy Thao (2015) has created a autologous fibrin and initially evaluated the effectiveness of the glue in the surgery to pterygium excision[3]. However, the procedure still not determined the rate of fibrinogen or thrombin, many chemicals required and quite complicated.

Thus, we have sought to make fibrin glue from rabbit plasma by a simple, easy and inexpensive method with the aim: Identified the process preparation of fibrin sealant by frozen plasma method.

METHODS

Participants, time and location of study

Research period: from January 2019 to March 2020.

Research materials:

The experimental study was carried on 05 healthy rabbits weighing 2000-2500 grams, not pregnancy, without coagulopathy.

Methods

Research design: experimental study

Research index:

Fibrinogen concentration in the control sample; Concentrated fibrinogen concentration after freezing at -18°C and -35°C at the different time: 2 hours, 12 hours; 24 hours and 48 hours.

Fibrinogen isolation

- Step 1: 20ml of rabbit's blood with anticoagulant and centrifuged.

. Tube 1: control tube is plasma without freezing.

. Tube 2: plasma freezing at -18°C for 2 hours.

. Tube 3: plasma freezing at -35°C for 2 hours.

After 2 hours, tube 2 and 3 defrosted at room temperature (26-28°C) and perform decantation of part of the plasma in 2 tubes to collect fibrinogen.

. The experiment was repeated on 5 blood samples of rabbit. Conclusion of fibrinogen concentration obtained after 2 hours at -18°C and -35°C (i).

- Step 2: The steps were similar to the first step, but freezing time is at 12 hours, 24 hours and 48 hours.

. The experiment was repeated on 5 blood samples of rabbit.

 Conclusion of fibrinogen concentration obtained after 12 hours, 24 hours and 48 hours at -18°C and -35°C (ii).

 From (i) and (ii) concluded the freezing time and temperature yield the highest amount of fibrinogen (*).

Simultaneous fibrin glue by separating fibrinogen and thrombin.

Based on the conclusion after fibrinogen extraction (*), the fibrin glue formation process was performed on a rabbit's blood sample according to the following procedure:

- Rabbit's blood put into the anticoagulation tube.

- Centrifuged and separated plasma into 2 tubes.

+ Tube 1: plasma used for isolation thrombin.

+ Tube 2: plasma used for isolation fibrinogen.

- Simultaneous coagulation of 2 tubes at the temperature and time was concluded after fibrinogen extraction (*).

- Defrosted 2 above tubes at room temperature (26-28°C).

- Collected thrombin

Tube 1: put $CaCl_2$ into plasma and collecting thrombin floating solution.

- Collected fibrinogen

Tube 2 (iiii): plasma was defrosted at room temperature and decant part of the plasma to obtain the remains.

- Creating fibrin glue

+ Mix 1 fibrinogen + 1 thrombin.

The experiment was repeated on 5 rabbit's blood samples.

 Conclusion: preparation of fibrin sealant by plasma frozen method.

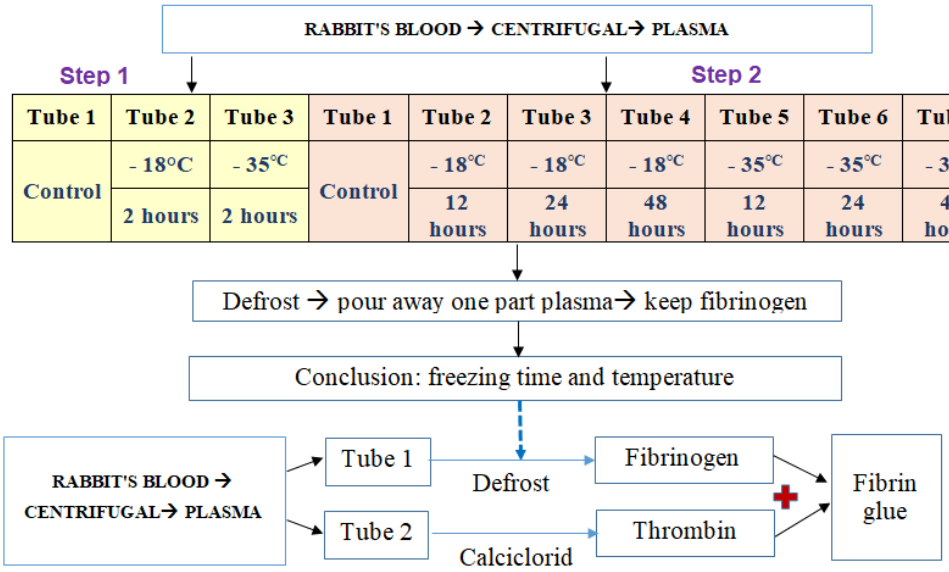

Figure 1. Research diagram

Equipment: Blood collection kits, clean, dry test tubes, Chinese Smic 80-2 inclined centrifuge, staRmax fibrinogen quantification machine from Stago-France.

RESULTS

Table 1. The concentration of fibrinogen (fib) was obtained after 2 hours at -18°C and -35°C

Samples	Controls (g/L)	- 18°C		- 35°C	
		g/L (1)	%	g/L (2)	%
1	1.05	0.26	24.76	0.26	24.29
2	2.20	1.07	*48.64*	0.70	31.59
3	4.12	1.19	28.88	1.24	30.10
4	2.17	1.00	45.85	0.83	38.25
5	2.35	0.20	08.51	0.73	30.85
$\overline{X}$ ± SD	2.38 ± 1.10	0.74 ± 0.47		0.75 ± 0.35	
p			1-2 > 0.05		

Table 1 shows that, the average concentration of fibrinogen obtained at temperatures of -18°C and -35°C was similar in each tube after 2 hours of freezing. The amount of fibrinogen obtained was from 08.51 to 48.64%.

Table 2. The concentration of fibrinogen (fib) was obtained over time causing coagulation at -18°C

Samples	Controls (g/L)	12 hours		24 hours		48 hours	
		g/L (1)	%	g/L (2)	%	g/L (3)	%
1	2.22	1.17	52.70	1.11	49.77	1.13	50.68
2	2.24	1.24	55.13	1.22	54.24	1.12	49.78
3	2.52	1.29	50.99	1.29	51.19	1.20	47.42
4	2.09	0.97	46.41	0.94	44.74	0.71	33.97
5	4.89	2.67	54.60	2.66	54.29	2.63	53.78
$\overline{X}$ ± SD	2.79 ± 1.18	1.47 ± 0.68		1.44 ± 0.69		1.36 ± 0.74	
p				1-2; 2-3; 1-3 > 0.05			

Table 2 shows that the average fibrinogen concentration obtained during freezing at -18°C did not have a significant difference (p>0.05) at 12 hours, 24 hours and 48 hours. The percentage of fibrinogen obtained when coagulating was in the range of 12 to 48 hours from 33.97 (at 48 hours) to 55.13% (at 12 hours).

Table 3. The concentration of fibrinogen (fib) was obtained over time to freeze at -35°C

Samples	Controls (g/L)	12 hours		24 hours		48 hours	
		g/L (1)	%	g/L (2)	%	g/L (3)	%
1	2.22	1.12	50.45	1.25	56.31	1.09	49.10
2	2.24	1.15	51.12	1.22	54.46	1.28	56.92
3	2.52	1.34	53.17	1.32	52.38	1.35	53.57
4	2.09	0.92	44.02	0.65	*31.10*	0.69	33.01
5	4.89	2.81	57.46	2.74	55.93	2.45	50.00
$\overline{X}$ ± SD	2.79 ± 1.18	1.47 ± 0.76		1.44 ± 0.78		1.37 ± 0.65	
p				1-2; 2-3; 1-3 > 0.05			

Table 3 showed that, the average fibrinogen concentration obtained during freezing at 35°C did not show a significant difference (p>0.05) at 12 hours, 24 hours and 48 hours. The percentage of fibrinogen obtained when coagulating ranges from 12 to 48 hours from 31.10 (at 24 hours) to 57.46% (at 12 hours).

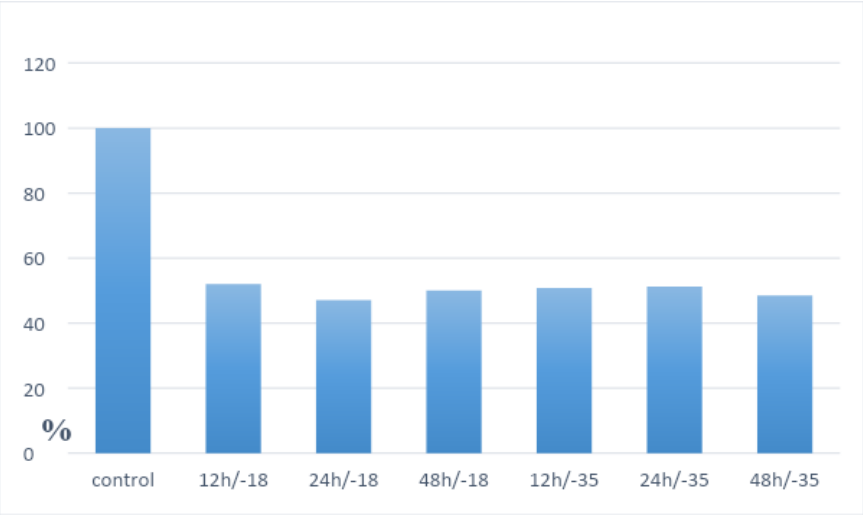

Figure 2. Compare the average fibrinogen ratio obtained at -18°C, -35°C when freezing at times (12, 24 and 48 hours)

Figure 2 shows that the average fibrinogen ratio obtained at -18°C, -35°C when freezing at 12, 24 and 48 hours did not show a significant difference (p>0.05).

Table 4. Time isolation thrombin and time creating fibrin glue

Sample	Isolation thrombin (s)	Creating fibrin glue (s)
1	96	86
2	157	103
3	242	183
4	193	152
5	141	162
$\overline{X}$ ± SD	165.80 ± 55.02	137.20 ± 41.00

Table 4 showed that, average thrombin collection time is 165.80 ± 55.02 seconds. The average self-fibrin formation time is 137.20 ± 41.00 seconds.

* The process of creating fibrin glues by freezing method itself was shown by the following diagram:

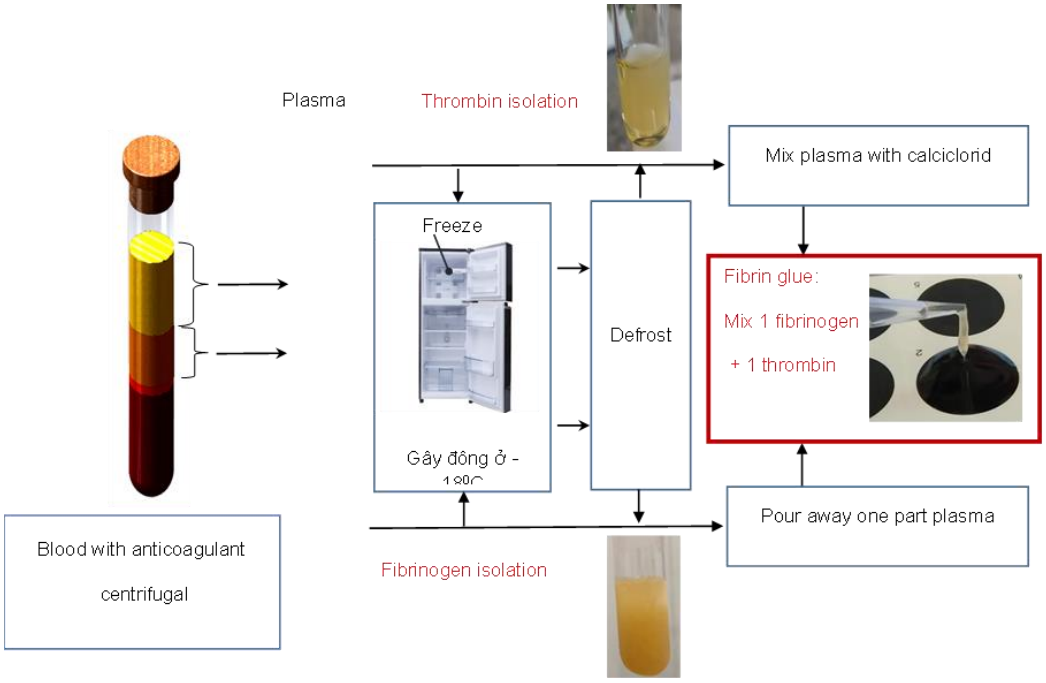

Figure 3. Process diagram of creating fibrin itself

DISCUSSION

Temperature, freezing time

Producing fibrin glue was based on "mimicking" the natural clotting mechanism to form a fibrin clot[4]. Studies have shown that the strength and bonding strength of the adhesive is directly related to the fibrinogen concentration[5-6]. Therefore, various methods of collecting fibrinogen itself have been tested. Techniques for using chemicals (ethanol, polyethylene glycol, ammonium sulfate) or using drugs (protamin) are quite effective and can lead to elevated fibrinogen[5-6]. But those methods require complex execution processes, expensive materials, chemicals and technicians must have skills. Therefore, we selected fibrinogen extraction method by plasma coagulation. The method aims to limit the amount of chemicals used and simple steps are easy to follow.

The process of fibrinogen extraction by plasma coagulation method is based on the use of deep negative temperature to cause reversible fibrinogen precipitation. In cold environments, the protein's secondary, tertiary, and quaternary structures are altered but not disrupt its primary structure, which is called protein denaturation. Denatured proteins are usually non-functional. Based on the properties of the fibrinogen after denaturation, the solubility decreases (the hydrophobic groups turn inside the protein molecule) and the ability to retain water decreases so that we cause blood clotting at -18°C and -35°C, and then defrost it. Decant the plasma containing a lot of water and retain the fibrinogen.

In order to determine the temperature point, the optimal freezing time to obtain the maximum amount of fibrinogen we chose the freezing time is 12 hours, 24 hours and 48 hours. Because according to the Ministry of Health's circular guiding blood transfusion regulations, a unit of plasma (250 mL) must be frozen for a maximum period of 8 hours and a temperature of -18^{0}C or less[7]. However, the amount of plasma needed for coagulation in our study was very small (1-2 mL), so it would coagulate faster than 1 plasma unit (250 mL), so we chose to explore further freezing time is 2 hours. Some studies also use freezing temperatures of -18°C[1]; or -20°C[8] ; or -80°C[9].

Research results in table 1; 2; 3; Figure 1 shows that, the average amount of fibrinogen obtained when causing plasma coagulation at 2 hours is lower than at 12 hours, 24 hours and 48 hours. Compared, the average amount of fibrinogen obtained at 3 times of freezing time was 12g, 24 hours and 48 hours with no significant difference. At the same time, the results also indicated that at the temperature of -18°C or -35°C, the amount of fibrinogen obtained was similar.

The interesting is that we just need to freeze the temperature in the freezer compartment of a freezer (-18°C). We need not a dedicated freezer cabinet. Time to cause plasma freezing should be at least 12 hours, and can be up to 48 hours.

Although there was no difference in the average fibrinogen concentration at the time of coagulation 12 hours, 24 hours and 48

hours, but the tables in 2 and 3 show that, the tendency to get the most fibrinogen concentration when freezing at 12 hours, and descending after 24 hours to 48 hours. This result is contrary to the initial hypothesis that the higher the fibrinogen concentration is obtained for a longer time. This may be because the method of plasma freezing has completely denatured fibrinogen (protein does not return to the state the first) and reduces the concentration of fibrinogen needed to make fibrin glue. Yoshida H. has shown that, the concentration and quantity of plasma components (including fibrinogen) stored at -20°C are significantly higher than those of plasma stored at - 80°C[9].

Compared to the research of Rock G. et al, the amount of fibrinogen obtained in our study is quite high[10]. The ratio of fibrinogen obtained when coagulating at 12 to 48 hours at -18°C (Table 2) is from 33.97 (at 48 hours) to 55.13% (at 12 hours); while at -35°C (Table 3) minimum 31.10% and a maximum of 57.46%. In study of Rock G.[10], the amount of fibrinogen obtained was 20 ± 2% (mean 2.2 ± 0.77 g / L) and 27% in another study with colleague Freedman M.[11]. There have been many studies confirming that, fibrinogen collection by cold precipitation is less effective than using protamin. Indeed, the fibrinogen results obtained in this study were lower than the levels of fibrinogen extracted by protamine in the study myself and colleagues[12] (the amount of fibrinogen obtained from 67.42% to 99.64%). So, this difference is probably due to the way plasma is mixed to quantify fibrinogen. In this study, we decant some of the plasma and carry the remaining plasma to quantify fibrinogen. While using protamin method, fibrinogen precipitate is mixed with the solution to precipitate then carry out quantitative fibrinogen. Huynh D. T. et al.[3] also used the freezing method to separate the components (fibrinogen and thrombin) from human plasma. However, the implementation process is quite complicated, the freezing temperature is -20°C for 1 hour, centrifuging 2 times, thrombin collection method using some additional chemicals such as acetic acid, pH adjustment ... The study did not provide a concentration of the glue and did not identify the factors affecting the glue production.

In summary, the time to freeze plasma is about 12-24 hours at -18°C (Figure 2), this is not too long, and can reduce the risk of infection.

Create fibrin

We create fibrin glue by the method of freeze plasma at -18°C for 24 hours. The results showed that (table 4), the time to create thrombin was 165.80 ± 55.02 seconds, the time to create fibrin was 137.20 ± 41.00 seconds. It takes longer to collect the components of the fibrin (thrombin, fibrinogen) glue than the create fibrin with protamin (15 minutes). However, the advantage in the procedure of the cryopreservation method is that, after 24-hour freezing, the formation of fibrinogen is very fast, the plasma after being removed from the refrigerator just needs to wait for thawing and decanting a part of plasma. While, the process of collecting fibrin glue with protamin is more complicated, the purchase of protamin is not easy, high cost, centrifugal to extract the precipitate, precipitate dissolved chemicals ...

Compared with the study of Vu T. K. L. et al.[2], the time to form fibrin glue in our study is faster than the glue formation time by the method of Vu T. K. L. (7 up to 9 minutes, average 8 minutes). The reason we mentioned above is that, after separating fibrinogen we use the same amount of plasma without mixing with other solutions, while in Lien's study was dissolve the precipitate with natricitrate solution. The presence of protamine and natricitrate in the glue may have diluted or affected the formation of glue. Inside, the blood sample in Lien's study was taken from humans. So, to confirm the difference in blood clotting time between rabbits and humans, more research is needed. The time for forming fibrin glue in our study is similar to that study of Huynh D. T.[3] (about 3 minutes).

Summary, we chose the simplest and most convenient procedure to create fibrin itself: plasma freezing time is from 12 to 48 hours at -18°C. Should choose a shorter time will reduce the risk of infection. May be isolation fibrinogen and thrombin according to usage needs.

So obviously with the method of creating glue by freezing although it is time-consuming, but the process is quite simple, low

cost, cheap equipment. Assuming the procedure is used on a patient, the subject only needs to take one blood sample just in combination with a blood sample to do other tests the day before. The fibrinogen extraction method does not require a centrifuge to separate the precipitate, the chemical is minimal, the time to create colloidal quickly, these help limit contact with the surrounding equipment environment to reduce the risk of infection. bacteria. The time to form the glue after mixing is not too fast to ensure enough time to perform graft adhesive during surgery.

CONCLUSION

Plasma freezing at a temperature of -18°C from 12-48 hours. Defrost and decant a portion of the plasma to collect fibrinogen. Creating thrombin by adding calcium chloride to the plasma. Mix fibrinogen with thrombin in a ratio of 1:1 to create fibrin glue.

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