

Preparation of fibrin glue: the effects of calcium chloride and sodium chloride

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Abstract

Concentrated fibrinogen is prepared from whole blood by cryoprecipitation and is then combined with a thrombin solution to make fibrin glue. Fibrin glue has been widely employed in many phases of surgery to control bleeding and to seal tissue defects. The purpose of this research was to study the effect of ionic strength of thrombin solutions on the properties of fibrin glue.

Fresh bovine plasma was frozen at -15°C for 24 h to prepare concentrated fibrinogen. The thrombin solution was prepared by reconstituting topical bovine thrombin with calcium chloride solutions to give a final concentration of 10, 20, 40, or 80 mM calcium chloride, with or without 0.9% w/v sodium chloride solution. The clotting time was measured by using a fibrometer and the bonding strength of fibrin glue was determined by measuring the force required to shear apart two collagen films joined by fibrin glue.

The results showed that faster gelation was obtained when 20–40 mM calcium chloride was used. In contrast, the addition of physiologic saline (0.9% w/v sodium chloride), slowed down the gelation of all samples. It is concluded that high bonding strength and rapid formation of fibrin glue can be obtained using 10 units ml^{-1} thrombin reconstituted with 20 mM calcium chloride solution, in the absence of sodium chloride.

Keywords: Fibrinogen; Fibrin glue; Cryoprecipitation

1. Introduction

Fibrin glue, a biological tissue glue derived from blood, is an effective hemostatic agent and sealant for tissue defects, and has been widely employed in many phases of surgery [1]. After application, fibrin glue is slowly reabsorbed over a period of days to weeks by fibrinolysis. The major component of fibrin glue is fibrinogen, which is a soluble protein present in blood and composes about 0.2% by volume of whole blood. The normal level of fibrinogen in blood plasma ranges from 2 to 4 mg ml^{-1} [2]. The major function of fibrinogen is to form a clot to stop bleeding. In the presence of thrombin and calcium ions, fibrinogen molecules are proteolytically cleaved and converted to fibrin monomers. Fibrin monomers assemble into fibrils and then fibers to form a three-dimensional, insoluble fibrin network, a fibrin clot, in the presence of calcium ions and Factor XIIIa. A fibrin clot is the final product of the normal coagulation cascade [3]. The fibrin glue system mimics this coagulation process and

is usually composed of two major components, a fibrinogen solution and a thrombin solution.

Autologous blood, single donor blood, or pooled homologous blood are commonly used to prepare concentrated fibrinogen solutions. Citrated blood is first centrifuged to remove the cells and then the plasma is treated with either physical or chemical precipitation methods to produce a concentrated fibrinogen solution [4]. Commercially available bovine thrombin is usually used as the second component. These two solutions are simultaneously ejected from syringes and mixed at the desired application site to form a gel.

There are several factors that affect the properties of fibrin glue: the precipitation method [5], the thrombin concentration [5], and the ionic strength of the fibrinogen and thrombin solutions [5]. Cryoprecipitation is considered to be the standard method for preparing fibrinogen; however, one freeze-thaw cycle can take more than 24 h. Chemical precipitation is considered a faster and easier way to prepare fibrinogen. The use of chemicals, such as ammonium sulfate, ethanol, and poly(ethylene glycol), have been reported [6–10].

In our laboratory, we have compared cryoprecipitation (cryo) to precipitation of fibrinogen using ammonium sulfate (AS), ethanol, and poly(ethylene glycol) (PEG) in terms of their fibrinogen yields, total protein yields, clotting time,

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and bonding strength [4]. The results suggest that AS precipitation is as effective as cryo in forming concentrated fibrinogen solutions and fibrin glue, and more effective than ethanol and PEG precipitation.

Commercial bovine thrombin is usually supplied as freeze-dried powder in a sterilized container and must be reconstituted to the desired concentration before use. Thrombin is commonly reconstituted using either sterile water, calcium chloride solution, or isotonic saline; however, the effects of these ions added to the fibrin glue system are not clear. The purpose of this research is to study the effects of calcium chloride and sodium chloride on the gelation time and bonding strength of fibrin glue. The results of this study showed that high bonding strength and rapid formation of fibrin glue can be obtained using 10 units ml^{-1} thrombin reconstituted with 20 mM calcium chloride solution, in the absence of sodium chloride.

2. Materials and methods

2.1. Isolation of fibrinogen

Fresh bovine blood, obtained at slaughter, was collected in a container containing sodium citrate buffer (10% w/v, pH 7.4) to give a final concentration of 1% w/v. The blood was centrifuged at 600g for 20 min, and then the plasma was carefully pipetted out. 10 ml of plasma was transferred to each of eight plastic tubes, frozen at -15°C for 24 h, and then thawed at 4°C . The thawed plasma was centrifuged at 3000g for 5 min and the supernatant discarded. The pellet was resolubilized in 1 ml distilled water, then centrifuged at 3000g for 5 min. The supernatant was retained as the soluble fraction and the pellet was discarded.

The protein concentration of the soluble fraction was determined by spectrophotometry at a wavelength of 280 nm. The optical density at $\lambda = 280$ nm was converted to concentration in mg ml^{-1} by multiplying the absorbance by 0.667 mg ml^{-1} . The fibrinogen concentration was determined by the clot collection method based on Ratnoff and Menzie [11] that was later modified [12].

2.2. Measurement of gelation time

A thrombin solution (20 units ml^{-1}) was prepared by reconstituting one vial of topical bovine thrombin (Thrombinar, 1000 units per vial, Armour Pharmaceutical Co., Kankakee, IL) with 50 ml distilled water. Calcium chloride solutions were prepared with concentrations of 20, 40, 60, 80, and 160 mM (with or without 1.8% w/v sodium chloride).

0.1 ml of concentrated fibrinogen solution was transferred to each of three test cups placed on the fibrometer. 0.1 ml of calcium chloride solution and 0.1 ml thrombin solution were transferred together to three additional test cups placed on the fibrometer to produce a final thrombin concentration of

10, 20, 40, and 80 mM (0.9% w/v sodium chloride). The solutions were allowed to warm up to 37°C for 3 min prior to testing. The thrombin and calcium chloride solution were then pipetted into the cup containing 0.1 ml concentrated fibrinogen solution. The timer was started at the end of ejection and the clotting time was recorded.

2.3. Fibrin glue preparation and shear strength testing

Collagen films, 4.5 cm \times 9.0 cm, were glued with epoxy to a vellum paper frame (7.0 cm \times 7.5 cm) which had borders measuring 1.0 cm in width. After the epoxy had dried, the film and the frame were cut into two equal halves. The two halves of the collagen film and paper frame were overlapped exactly 1.0 cm. The paper frame was then rejoined with tape ensuring that the overlapped ends of the collagen films remained unrestrained. Using the fibrin sealant applicator system, 0.15 ml of soluble fibrinogen solution and 0.15 ml of thrombin solution were simultaneously injected onto the overlapped area of the films. The fibrin glue was carefully pressed into an even layer between the films with a 1.0 kg weight. The fibrin sealant bound films were placed in a humid atmosphere and allowed to cure for 30 min at 37°C . After curing for 30 min, the films were removed from the incubator and cut into strips 1.0 cm in width. The strips were tested in uniaxial tension at a strain rate of 5 mm min^{-1} using an Instron Model 1122 mechanical tester. The shear strengths of the fibrin glue were calculated from the load/elongation curves. The statistical variation between samples was determined using a Student's *t*-test ($p \leq 0.05$).

3. Results

3.1. Clotting times

Fig. 1 summarizes the results of the clotting time experiments conducted as a function of CaCl_2 concentration with and without the addition of physiologic saline (0.9% w/v NaCl) to the thrombin solution. The experimental results indicate that in the absence of sodium chloride, the clotting time of the cryoprecipitate gradually decreased from 5.7 s to 2.3 s with the addition of up to 20 mM calcium chloride to the thrombin solution (10 units ml^{-1}). The clotting time curve did not change from 20 to 40 mM of calcium chloride, but when 80 mM calcium chloride was added to the thrombin solution the clotting time increased to 4.7 s. When 0.9% w/v sodium chloride was added with calcium chloride to the reconstituted thrombin solution, the clotting time of all samples increased significantly.

3.2. Shear strength of fibrin glue

Fig. 2 summarizes the results of the shear strength experiments conducted as a function of CaCl_2 concentration with and without the addition of 0.9% w/v NaCl to the thrombin

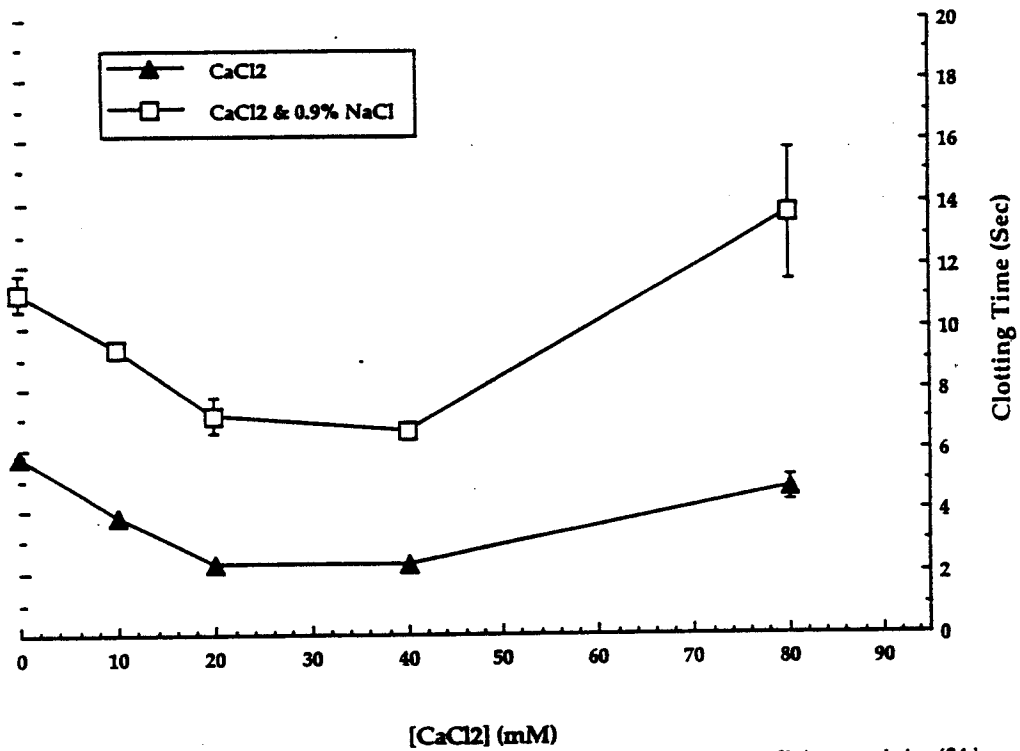


Fig. 1. Effect of calcium chloride and sodium chloride solution on the clotting time of fibrin glue prepared from fibrinogen solution (24 h cryoprecipitation) and thrombin solution (10 units ml^{-1}).

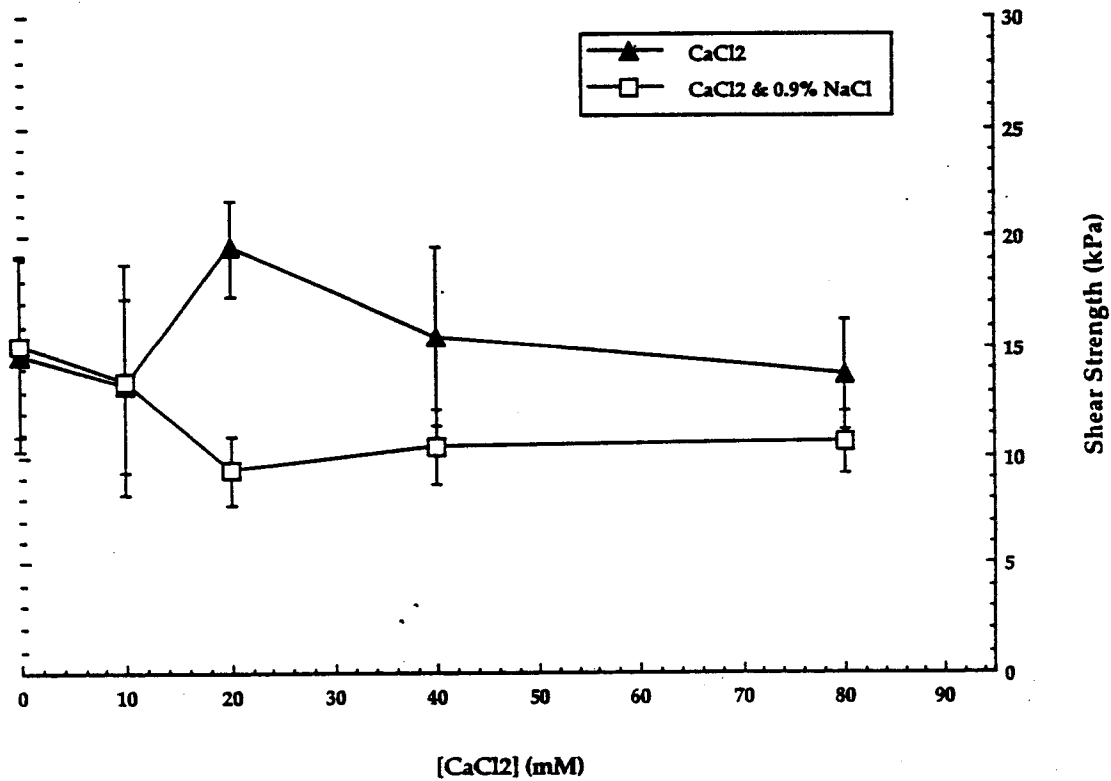


Fig. 2. Effect of calcium chloride and sodium chloride solution on the bonding strength of fibrin glue adhered to collagen films. The glue was prepared from fibrinogen solution (24 h cryoprecipitation) and thrombin solution (10 units ml^{-1}).

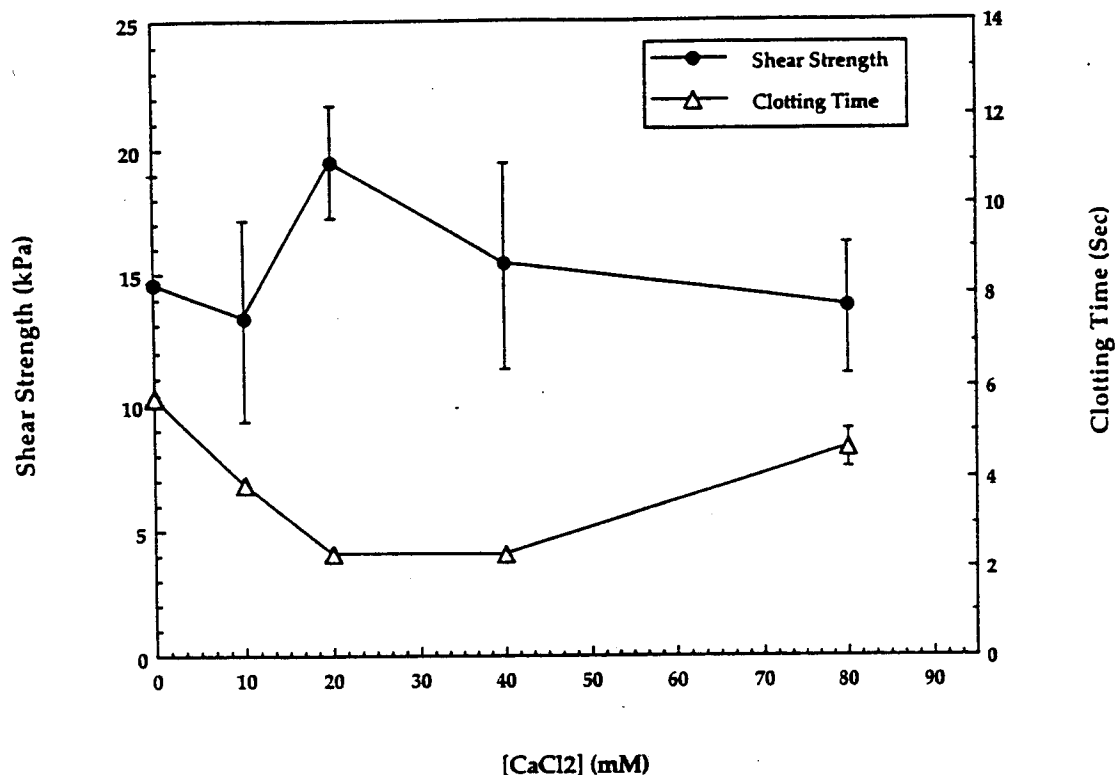


Fig. 3. A summary of the effect of calcium chloride on the bonding strength and clotting time of fibrin glue prepared from fibrinogen solution (24 h cryoprecipitation) and thrombin solution (10 units ml^{-1}).

solution. The shear strength of the fibrin glue made with cryoprecipitated fibrinogen and thrombin solution (10 units ml^{-1}) reconstituted with distilled water was about 14.6 kPa. The shear strength of the fibrin glue samples containing 20 mM calcium chloride and no sodium chloride was 19.5 kPa, which was significantly higher than the shear strength of other samples. Fibrin glue samples made with 20–80 mM of calcium chloride in the thrombin solution exhibited significantly higher shear strengths than comparable samples containing 0.9% sodium chloride.

4. Discussion

Fibrin glue has been used in many clinical applications, such as ophthalmic surgery, plastic reconstruction, drug delivery, cardiovascular surgery, orthopedic surgery, and neurosurgery [5]. In most cases its ability to form strong tissue adhesion as rapidly as possible is important for its applications. In this paper we studied the effects of calcium chloride and sodium chloride on the bonding strength of fibrin glue to collagen films. Collagen films were made from bovine type I collagen. Type I collagen was chosen because it is the major structural component of most connective tissues and organs.

Using a lap shear test, we have previously determined the bonding strength of fibrin glue prepared from cryoprecipitation and chemical precipitation methods using 10 and 200 units ml^{-1} thrombin [1]. The results showed that fibrin glues

made from 10 and 200 units ml^{-1} thrombin had comparable bonding strengths. However, at low thrombin concentrations, i.e. 10 units ml^{-1} , the gelation of fibrin glue was relatively slow, with clotting times ranging from about 4 s for cryoprecipitates to 13 s for ammonium sulfate precipitates. In contrast, when a high thrombin concentration (200 units ml^{-1}) was used, the clotting time was reduced to 1.3 s.

In this study, using 10 units ml^{-1} thrombin, we found that the clotting time of 24 h cryoprecipitate was reduced to 2.3 s when 20–40 mM of calcium chloride was added to the thrombin solution. In addition, the results of the lap shear test showed that the shear strength of fibrin glue was highest for samples containing 20 mM of calcium chloride.

In vivo, in the final stage of the normal coagulation cascade, fibrinogen molecules are converted into fibrin monomers by thrombin in the presence of calcium ions. Fibrin monomers are then assembled and cross-linked into an insoluble fibrin network by the formation of isopeptide bonds in the presence of Factor XIIIa and calcium ions. Factor XIIIa is a transglutaminase present in blood and its activity is calcium ion-dependent [2]. After coagulation, Factor XIII, a zymogen, is cleaved by thrombin, to completely expose the active center. To obtain maximal activity, calcium ions are required. In summary, calcium ions facilitate the conversion of fibrinogen molecules to fibrin and enhance the activity of Factor XIIIa, which helps stabilize the three-dimensional structure of insoluble fibrin networks. The interactions of calcium ions with the coagulation proteins are probably the reason why the

bonding strength of fibrin glue was increased and the gelation time was decreased when 20 mM of calcium chloride were added to the thrombin solution (see Fig. 3).

In contrast, sodium ions had the opposite effect on the fibrin glue system. The addition of 0.9% w/v sodium chloride slowed down the gelation of all samples (see Fig. 1) and offset the increase in shear strength caused by the addition of 20–80 mM calcium chloride. As indicated by a recent article [13], when the ionic strength is increased, the fibrin diameters and pore sizes of the fibrin gel are reduced, resulting in the formation of a thinner gel. Thinner fibrin fibrils would be expected to give a lower shear strength glue under these conditions, as was observed in our study.

The amount of thrombin used to make fibrin glue for the treatment of severely traumatized and burned patients is an important consideration. The human coagulation system is capable of responding to the severity of body injuries. After trauma, fibrinogen and platelet concentration in the body are elevated and the body is in a state of hypercoagulability [14,15], where disseminated intravascular coagulation is likely to occur [16]. When thrombin enters into the blood circulation, platelet and fibrin start to deposit in capillaries, arterioles, and venules of various organs, and sometimes thrombosis occurs. In animal studies, thrombin infusion caused pulmonary embolization [17,18], increased pulmonary vascular permeability to proteins [17–19], pulmonary edema [17,18], PMNs accumulation in the pulmonary circulation [19], and neutrophil-dependent pulmonary vascular injury [20,21]. In addition, fibrin deposition in the pulmonary circulation is associated with the disruption of microvascular endothelium [19,21], and the degradation products of fibrin can cause pulmonary edema. Therefore, caution should be taken in the use of fibrin glue for hemostasis in severe traumatized or burned patients, and the use of a lower concentration of thrombin would probably reduce the risk of these potential adverse reactions.

5. Conclusions

The results of our previous study showed that the bonding strengths of fibrin glue are similar for samples using 10 and

200 units ml⁻¹ thrombin. In this study it was shown that the gelation time can be reduced to 2.3 s when 10 units ml⁻¹ thrombin is reconstituted with 20–40 mM calcium chloride. High bonding strength and rapid formation of fibrin glue can be obtained using 20 mM calcium chloride solution, no sodium chloride, and 10 units ml⁻¹ thrombin.

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