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Performance Evaluation of Autologous Thrombin Produced from Platelet-rich Plasma (PRP) Tubes

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Abstract: Thrombin derived from bovine sources is commonly used to arrest bleeding during surgical procedures. However, there are risks associated with the use of bovine-derived thrombin, such as postoperative bleeding and the risk of infection in patients. Therefore, it is essential to develop a technology to generate autologous thrombin. In this study, autologous thrombin was produced from platelet-poor plasma (PPP) obtained using PRP tubes, mixed with 10% calcium gluconate, and halloysite nanotubes (HNTs), and evaluated the stability of prepared thrombin when stored at room temperature. The experiment demonstrated that the combination of 3 ml of platelet-poor plasma (PPP), 2.3 ml of 10% calcium gluconate, and a minimum of 1.5 mg of HNTs produces autologous thrombin with enhanced activity and stability, providing experimental evidence for its potential clinical application.

Keywords: autologous thrombin; stability; 10% calcium gluconate; platelet-poor plasma; halloysite nanotubes

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1. Introduction

Thrombin is a serine protease, which is very important for the regulation of the coagulation cascade. The production of thrombin is initiated by the formation of prothrombinase complex on the surface of endothelial cells and activated platelets. The prothrombinase enzyme complex is composed of platelet phospholipids, calcium ion (Ca^{2+}) and coagulation factors Va and Xa. The complex converts prothrombin into thrombin through proteolysis[1].

For decades, thrombin has been used in surgery to reduce traumatic bleeding. In 1977, Jasani reported that 15 patients who received thrombin treatment after abdominal surgery had significantly less hematomas than the control group who did not receive thrombin treatment [2]. In addition, thrombin has also been successfully used in other fields, such as controlling bleeding during skin transplantation for burn patients [3] and heart surgery [4].

So far, thrombin used in surgery mainly comes from cattle. The use of bovine thrombin is potentially dangerous. For example, studies have shown that the use of bovine thrombin may lead to the formation of antibodies. These antibodies may form a cross reaction with coagulation Factor V (FV) of the patient, causing a series of symptoms, which may lead to adverse reactions, including severe and life-threatening bleeding [5]. Another concern is that they may be exposed to bovine-derived prions, causing humans to suffer from variant Creutzfeldt-Jacob disease (vCJD)[6]. Therefore, it is very necessary to produce human thrombin, preferably autologous thrombin, for surgery. Autologous thrombin has obvious

safety advantages, because it does not have the potential safety hazard of pathogens caused by plasma-related derivatives, and it also avoids the immunogenicity risk caused by contact with recombinant deoxyribonucleic acid (DNA) proteins of animal origin.

The simplest way to prepare autologous thrombin is to add calcium ion to the autologous citrated plasma. Excess calcium will start the coagulation cascade reaction and produce thrombin. However, the disadvantage of this method is the poor stability of the thrombin activity produced [7].

To address the stability problem of generated thrombin, this paper utilizes platelet-poor plasma (PPP) prepared from PRP tubes, 10% calcium gluconate, and halloysite nanotubes (HNTs) to prepare autologous thrombin. Halloysite nanotubes (HNTs) are the tubular forms of halloysite and are chemically similar to kaolin. They are layered aluminosilicates ($Al_2Si_2O_5(OH)_4 \cdot nH_2O$) with hollow tubular structure and high aspect ratio. The outer diameter is about 40–70 nm, the inner diameter is 10–20 nm, and the length is 500–1500 nm [8]. Because HNTs has a large surface area, positively charged inner surface (Al-OH groups) and negatively charged outer surface (Si-OH) and (Si-O-Si) groups, they can effectively bind with many biological molecules [9].

The structural analysis of thrombin showed that its two exosites (Exosite I and Exosite II) of thrombin showed obvious positive potential, while the center of the active site showed negative potential, forming a "Positive-negative-positive" charge pattern[10].

Nor-chelated surplus calcium in citrated plasma will start the coagulation cascade reaction and produce thrombin. Moreover, HNTs' unique tubular structure and high aspect ratio make it have a large specific surface area and pore volume, so that its outer surface carries more negative charges, which can better activate factor XII and other substances in plasma to convert into thrombin [11]. Thrombin exhibits a characteristic "positive-negative-positive" charge distribution, whereas halloysite nanotubes (HNTs) possess a high specific surface area with a positively charged inner lumen and a negatively charged outer surface. Consequently, thrombin molecules are immobilized on both the inner and outer surfaces of HNTs through electrostatic interactions and physical adsorption, which markedly improve the stability of thrombin.

This paper explores the effects of calcium ion concentration and the amounts of halloysite nanotubes (HNTs) on the activity and stability of autologous thrombin. By controlling the volume ratio of 10% calcium gluconate to platelet-poor plasma (PPP) and varying the amounts of added halloysite nanotubes (HNTs), we can determine the optimal parameters for the preparation of high activity and high stability autologous thrombin.

2. Materials and methods

2.1. Experimental materials and reagents

The materials and reagents used in this study are as follows: the autologous platelet-rich plasma (PRP) collection tube was manufactured by Zhuhai Longtime Biological Technology Co., Ltd. (the suction volume was 10 ml); 10% calcium gluconate injection was provided by Shanxi Ruicheng Kelong veterinary drug Co., Ltd; Halloysite nanotubes (HNTs, purity>98%) were purchased from Guangzhou Runhuo Material Technology Co., Ltd; Fibrinogen was purchased from Sigma Aldrich (sigma F8630); Thrombin was purchased from Hunan Yige Pharmaceutical Co., Ltd. The main reagent for thrombin activity detection in the experiment is fibrinogen solution, which is prepared by dissolving the fibrinogen powder in ultrapure water. All chemical reagents are analytically pure, and the experimental water is ultrapure water.

2.2. Experimental equipment

The main instruments and equipment used in this study are shown in **Table 1**:

Table 1. Experimental instruments and equipment

Device Name	Model	Purpose	Manufacturer
Coagulation Analyzer	TS6000	Thrombin Activity Detection	Med Pacific (Tianjin) Biotechnology Co., Ltd.
Planar Centrifuge	TDL-50B	Sample Separation and Preparation	Anting Scientific Instrument Factory
Constant Temperature Incubator	KLX-100B	Sample Incubation and Reaction	Jiangsu Claix Biotechnology Co., Ltd.
Vortex Mixer	SCI-VS	Sample Homogeneous Mixing	Beijing Dragon Lab Instrument Co., Ltd.
Analytical Balance	FA2004	Precise Reagent Weighing	Shanghai Precision & Scientific Instrument Co., Ltd.
Pipette	Various Specifications	Precise Liquid Transfer	Eppendorf (Germany)

All the instruments used in this experiment have been calibrated. The experimental ambient temperature was maintained at 25 ± 2 ℃ and the humidity was controlled at 50 ± 10%, ensuring the consistency of experimental conditions and the reliability of results.

2.3. Test method

2.3.1. Method for Preparing PPP from PRP Tubes

The PRP tubes produced by Zhuhai Longtime Biological Technology co., Ltd. were used to draw the blood of five volunteers, six tubes for each. After the blood draw was completed, turn the PRP tube upside down for 8 - 10 times to make the anticoagulant in the tube evenly mixed with the blood, then the tubes were subsequently centrifuged using a dedicated centrifuge supplied by Zhuhai Longtime Biological Technology Co., Ltd. The centrifugation condition is 1500 g*9 min. After centrifugation, the layers in the tube from top to bottom were: platelet-poor plasma (PPP), buffy coat, separation gel, and red blood cells, and then use the syringe to extract PPP.

2.3.2. Detection method of autologous thrombin activity

The activity of autologous thrombin was detected by Clauss method [12]. First, make the standard curve of thrombin activity. The method is as follows: prepare 2.0 mg/ml fibrinogen solution, incubate 200 μl fibrinogen solution at 37 ℃ for 3 minutes, and then add 100 μl thrombin solution of known concentration (30 IU/ml, 15 IU/ml, 20IU/ml, 10 IU/ml, 5IU/ml, 2.5 IU/ml, 1.25IU/ml). Use the TS6000 coagulation analyzer to record the time of clot formation. Each value is tested twice. The average value is the time that the thrombin coagulates fibrinogen. The standard curve of thrombin activity is as follows:

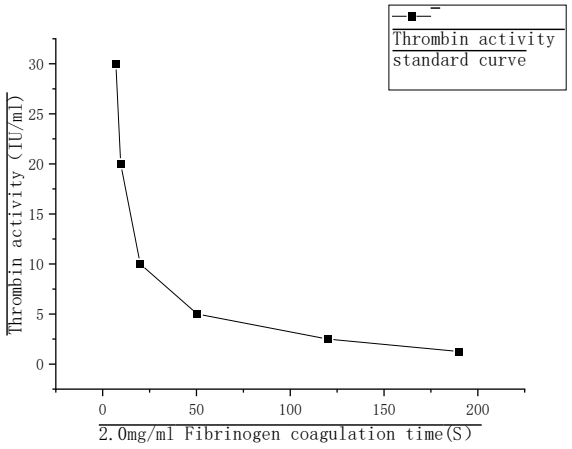

Figure 1. Standard curve of thrombin activity

Because each time point on the standard curve corresponds to the activity of thrombin that coagulates fibrinogen, the activity of thrombin in the unknown sample can be inferred from the coagulation time of fibrinogen.

2.3.3. Experimental process

Investigation of the influence of calcium concentration on autologous thrombin activity

Preparation of autologous thrombin

The experiment was divided into six groups: Fixed 3 ml PPP from PRP tubes, and 1.5 mg HNTs was added. The volume of calcium gluconate in groups 1 to 6 is 1) 3 ml 2) 2.3 ml 3) 1.8 ml 4) 1.5 ml 5) 1 ml 6) 0.6 ml (the volume ratios of 10% calcium gluconate to PPP in groups 1 to 6 are as follows: 1:1, 1:1.3, 1:1.6, 1:2, 1:3, 1:5)

Add the above samples, vortex for 2 minutes, incubate at 37 ℃ for 30 minutes, and finally centrifuge at 3500 g * 10 min to obtain the supernatant (autologous thrombin, ATS)

Autologous thrombin was prepared from the PPP of five volunteers according to the aforementioned method and incubated at room temperature for 0, 1, 2, 3, and 4 hours. 200 μ l of 2.0 mg/ml fibrinogen solution was incubated at 37 ℃ for 3 minutes in TS6000 coagulation analysis, subsequently, 100 μ l of the corresponding autologous thrombin was added, and the coagulation time was recorded by TS6000 coagulation analyzer. Detect twice at each time point, and take the average value, which is the time of coagulation of fibrinogen solution with autologous thrombin prepared at that time point.

Investigation of the influence of halloysite nanotubes (HNTs) amounts on the activity of autologous thrombin

By studying the effect of calcium concentration on thrombin activity, it was found that thrombin activity was highest when the volume ratio of 10% calcium gluconate to PPP was 1:1.3. Consequently, this 1:1.3 ratio was used in all subsequent experiments to evaluate how the amounts of halloysite nanotubes (HNTs) affect the activity of autologous thrombin.

Preparation of autologous thrombin

The experiment was divided into five groups: 2.3 ml of 10% calcium gluconate was added to 3 ml PPP, and then different amounts of HNTs were added to the respective groups as follows:

1(0mg 2(0.8mg 3)1.5mg 4)2.3mg 5)3.0mg

Add the above samples, vortex for 2 minutes, incubate at 37 ℃ for 30 minutes, and finally centrifuge for 3,500 g * 10 min to obtain the supernatant autologous thrombin.

Autologous thrombin was prepared from the PPP of five volunteers according to the aforementioned method and incubated at room temperature for 0, 1, 2, 3, and 4 hours. 200 μ l 2.0 mg/ml was incubated at 37 ℃ for 3 minutes in TS6000 coagulation analysis, subsequently, 100 μ l of the above autologous thrombin was added, and the time required for coagulation was recorded by TS6000 coagulation analyzer. Detect twice at each time point, and take the average value, which is the time of coagulation of fibrinogen solution with autologous thrombin prepared at that time point.

3. Experimental results

3.1. Effect of calcium concentration on thrombin activity

When the volume ratio of 1.10% calcium gluconate to PPP was 1:1.3, the prepared autologous thrombin activity was the highest; When the volume ratio of 10% calcium gluconate to PPP was 1:1 and 1.6, the thrombin activity was equivalent; When the volume ratio of 10% calcium gluconate to PPP was 1:2, 1:3 and 1:5, the thrombin activity decreased significantly.

The volume ratio of 10% calcium gluconate to PPP is 1:1. After 2 minutes of vortex, incubate at 37 ℃ for 30 minutes, and finally centrifuge at 3,500 g * 10 min. The gel formed after centrifugation is very soft. Many ATS are sandwiched in the gel, which is difficult to separate by centrifugation, Consequently, there is almost no supernatant after centrifugation. When pipetting ATS, it is easy to clog the 200 μ l pipette tip. After the experiment was completed, the gel formed had not

changed, and it is easy to clog the pipette tip when pipetting ATS.

When the volume ratio of 10% calcium gluconate to PPP is 1:1.3, the thrombin activity is highest. Following vortexing for 2 minutes, then incubated at 37 ℃ for 30 minutes, and centrifuged at 3500 g *10 min finally. This process effectively separates ATS from the gel, indicating that this is the optimal volume ratio of 10% calcium gluconate to PPP.

Table 1 and **Figure 2** present the time-dependent activity of autologous thrombin prepared with different volume ratios of 10% calcium gluconate to PPP.

Table 1. The effects of varying the 10% calcium gluconate-to-PPP volume ratios on autologous thrombin activity overtime

After ATS preparation	0h	1h	2h	3h	4h
10% calcium gluconate: PPP (volume ratio)					
1:1	7.57	7.02	6.84	6.65	6.43
1:1.3	10.3	8.13	7.84	7.41	7.24
1:1.6	8.77	6.93	6.72	6.44	6.06
1:2	5.52	3.67	3.44	2.86	2.61
1:3	Below Detection Limit	Below Detection Limit	Below Detection Limit	Below Detection Limit	Below Detection Limit
1:5	Below Detection Limit	Below Detection Limit	Below Detection Limit	Below Detection Limit	Below Detection Limit

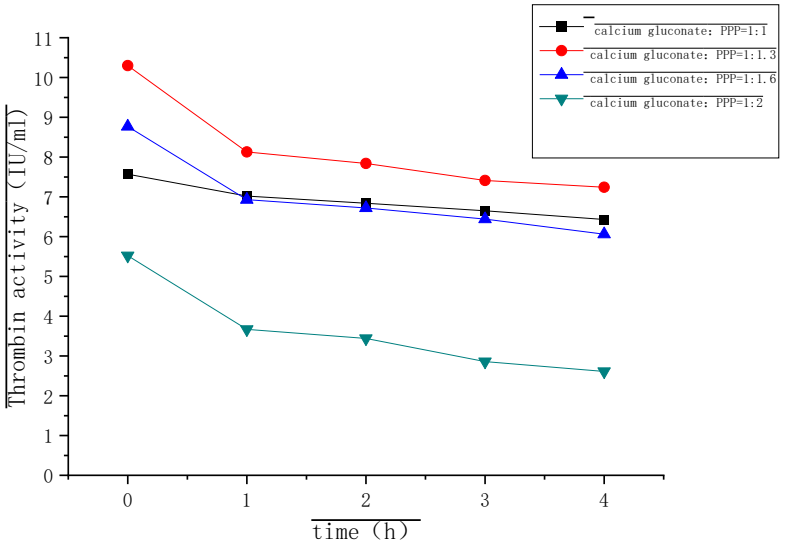

Figure 2. Influence of different 10% calcium gluconate-to-PPP volume ratios on autologous thrombin activity over time

Figure 3 shows the autologous thrombin tubes arranged from left to right, prepared with volume ratios of 10% calcium gluconate to PPP were 1:1, 1:1.3, 1:1.6, 1:2, 1:3 and 1:5, respectively, captured 4 hours post-experiment. As demonstrated in Figure 3, when 10% calcium gluconate solution was mixed with PPP at a 1:1 volume ratio, the resulting gel exhibited a very low stiffness and failed to form a pellet under centrifugation conditions (3500 × g for 10 min). When autologous thrombin is pipetted, it is easy to clog the pipette tip.

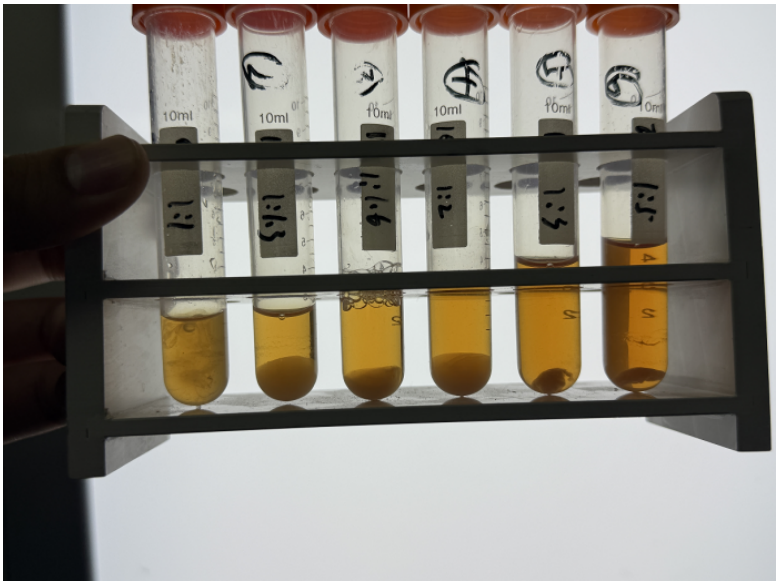

Figure 3. Image of autologous thrombin tubes after 4 h preparation, illustrating the influence of different volume ratios of 10% calcium gluconate to PPP

3.2. Effect of the amounts of halloysite nanotubes (HNTs) on the activity of autologous thrombin

When the amounts of halloysite nanotubes (HNTs) are equal to or exceed 1.5 mg, the prepared thrombin activity is high, and with the increase of time, the thrombin activity decreases slowly. (good stability)

When the amounts of HNTs are equal to or exceed 1.5 mg, the thrombin activity of the preparation remains statistically comparable at every measured time point within the 0–4 h interval.

Without HNTs, only PPP and 10% calcium gluconate were mixed to obtain thrombin with high activity at first (0 h), but the activity decreased rapidly (poor stability).

Table 2 and **Figure 4** summarize the time-dependent activity of autologous thrombin generated with different amounts of HNTs

Table 2. Time-dependent activity of autologous thrombin prepared with different amounts of HNTs

	Thrombin activity (IU/ml)				
After ATS preparation	0h	1h	2h	3h	4h
amount of HNTs added (mg)					
0.0	12.9	7.41	6.43	6.11	5.35
0.8	9.43	7.95	6.72	6.48	5.95
1.5	10.3	8.13	7.84	7.41	7.24
2.3	11.3	8.13	7.87	7.24	7.04
3.0	10.1	8.23	8.10	7.95	7.44

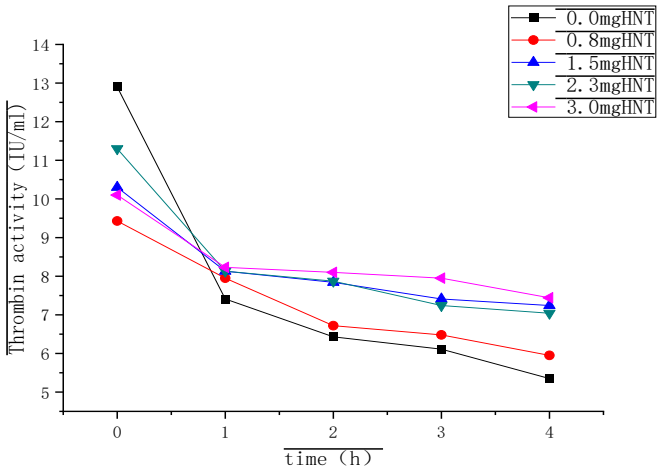

Figure 4. Time-dependent activity of autologous thrombin prepared with different amounts of HNTs

4. Discussion

Through a series of experiments, the key effects of calcium ion concentration and the amounts of halloysite nanotubes (HNTs) on the activity and stability of autologous thrombin were discussed, leading to several insightful conclusions.

First, in terms of the effect of calcium concentration, we found that the volume ratio of 10% calcium gluconate to PPP was one of the core factors determining the activity of autologous thrombin. The experimental results showed that when the volume ratio was 1:1.3, the prepared autologous thrombin activity was the highest. This ratio is better than the ratio of 1:1, 1:1.6 and more than the low calcium conditions of 1:2, 1:3 and 1:5. Under low-calcium conditions, thrombin activity declines dramatically, even falling below the assay's detection limit. It is worth noting that when the ratio is 1:1, although the initial activity is high, the texture of the gel formed is too soft, which makes it difficult to separate the supernatant (ATS) after centrifugation, and it is very easy to clog the pipette tip, which seriously affects its practical operability. Therefore, the 1:1.3 volume ratio demonstrates excellent activity and achieves the optimal balance between experimental feasibility and the purity of the separated product, thereby making it an ideal addition ratio.

Secondly, we investigated the regulatory effect of HNTs on the stability of autologous thrombin. Under the premise of fixing the optimal calcium ratio (1:1.3), the introduction of HNTs significantly improved the stability of autologous thrombin. The experimental data showed that when the added amounts of HNTs were equal to or exceeded 1.5 mg, the prepared thrombin not only had high activity, but also decreased slowly during the observation period of 0-4 hours, indicating markedly improved stability. In contrast, the control group without HNTs exhibited higher initial thrombin activity ; however, this activity decreased steeply over time, reflecting its poor stability.

It is mainly because thrombin has a "positive-negative-positive" charge pattern, and HNTs has a large surface area, positive inner surface and negative outer surface, so the thrombin formed is retained on the inner and outer surfaces of HNTs through charge and physical adsorption, so as to improve the stability of thrombin.

5. Summary

In summary, this study has successfully established an optimized autologous thrombin-preparation protocol: 2.3 mL of 10% calcium gluconate is added to 3 mL PPP, followed by the incorporation of at least 1.5 mg of HNTs. This protocol can produce autologous thrombin with high activity and high stability, which provides an experimental basis for its clinical transformation and application.

Disclosure statement

The author declares no conflict of interest.

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