JECT. 2008;40:94–98
The Journal of The American Society of Extra-Corporeal Technology

Autologous Thrombin: Intraoperative Production From Whole Blood

Vijay Kumar, PhD; John R. Chapman, PhD

Thermogenesis Corporation, Rancho Cordova, California

Presented at the 45th International Conference of the American Society of Extra-Corporeal Technology, Atlanta, Georgia, April 25–28, 2007.

Abstract: Thrombin is routinely combined in surgical practice with a fibrinogen source to prepare fibrin sealant to promote hemostasis or with platelet concentrates to prepare platelet gels to enhance wound healing. The purpose of this study was to evaluate the robustness and reproducibility of a new sterile handheld disposable thrombin-processing device (TPD) to generate autologous human thrombin in the intraoperative setting, using whole blood as the starting source material. By using whole blood instead of plasma as the starting material, it is possible to eliminate the plasma separation step from whole blood and reduce the thrombin production time and increase its availability to the surgical team intraoperatively. Active thrombin was prepared by combining 4 mL of thrombin reagent (a mixture of calcium chloride and ethanol) to 11 mL of blood in a reaction chamber containing negatively charged particles. The whole blood, reagent and particle mixture was incubated for 25 minutes at either 18°C or 24°C (n = 25/group) to assess stability of the thrombin activity. The mean activity of the thrombin produced at 18°C and 24°C was 52 ± 14 (n = 25) and 61 ± 12.2 IU/mL (n = 25), respectively. The average volume of thrombin harvested from each aliquot of blood at 18°C and 24°C was 10 ± 0.4 and 9 ± 0.6 mL, respectively. The thrombin concentration generated was shown to rapidly (<5 seconds) coagulate fibrinogen concentrate and retained clotting activity for 1 hour at room temperature (18–26°C) and up to 4 hours when stored on ice. The results show that the TPD is able to consistently generate high thrombin activity from human whole blood. The device offers a robust and rapid approach for preparing active thrombin from whole blood.
Keywords: blood, human thrombin, stability, fibrinogen, platelet-rich plasma, clot. *JECT. 2008;40:94–98*

Thrombin (activated factor II) is a serine protease that converts soluble fibrinogen to an active form that assembles into fibrin (1). Thrombin has been widely used in various surgical procedures to promote hemostasis in combination with collagen sponges (2–5) and with a concentrated fibrinogen source as an adjunct to achieve hemostasis (6). The known effect of thrombin on platelet function and clot formation has led to its use with platelet concentrate to form platelet gels (7). A number of studies on platelet gel have been produced for orthopedic, oral, and maxillofacial surgery as a means to enhance wound healing (8,9). It is proposed that bone growth can be enhanced because of an increased level of tissue growth factors associated with platelets granules (10,11).

The source material to derive thrombin used in surgical procedures is extremely important for the resulting thrombin risk profile to the patient. However, the majority of the thrombin used today is of bovine origin, and an immunologic response elicited by bovine thrombin preparations and their clinical sequela is well established (12). Because bovine thrombin preparations are a xenobiotic and not completely pure, an immunologic response can be elicited by bovine plasma contaminants such as factor V (13). The most serious adverse effects are mediated by antibodies with cross-reactivity to human factor V, which can cause a range of coagulapathy-associated symptoms that may lead to adverse reactions, including severe and life-threatening bleeding. Another concern particular for thrombin sourcing from bovine and pooled human products is possible exposure to prions causing variant Creutzfeldt-Jacob disease (vCJD) in humans (14). Thus, the patient's own blood is accepted as the safest source material for preparing thrombin used in surgical procedures.

The majority of the commercially available thrombin preparations are derived from large pools of mixed donor plasma by Cohn-Oncley fractionation (15). Currently, purified prothrombin preparation and different methods in-

Address correspondence to: Vijay Kumar, ThermoGenesis Corp., 2711 Citrus Road, Rancho Cordova, CA 95742. E-mail: vkumar@thermogenesis.com
The authors have reported a financial relationship with the entity whose products or services are discussed in this paper.

cluding use of different coagulation proteins (16,17) or snake venom as activators (18) were used to produce active thrombin. These methods are well adapted for industrial-scale production of thrombin from large pools of plasma. These are not practical methods for the preparation of single donor autologous thrombin.

The authors have previously reported the development of a simple and reliable method for development of autologous thrombin from single donor plasma (19). Thrombin production can be readily initiated by adding an excess of calcium ions to citrated plasma; however, the use of thrombin prepared in this simple manner is of limited value because of the slow rate of thrombin production and the low concentration of thrombin produced. The use of glass beads in combination with thrombin-processing device (TPD) reagent in the current method overcomes these limitations and greatly accelerates the rate of thrombin production and stabilizes the thrombin by blocking the inhibitory actions of protein C, protein S, and, particularly, anti-thrombin III (1). The resulting thrombin can be stored for hours after production and maintain clinical use.

Recently, we optimized the process for producing thrombin from whole blood intra-operatively, followed by centrifugation (20). In this study, we developed a robust procedure to generate autologous human thrombin in the intraoperative setting from whole blood instead of plasma as the starting source material within a 30-minute period. By using whole blood instead of plasma as the starting material, we eliminated the plasma separation step and reduced the thrombin production time, thereby increasing its availability to the surgical team intraoperatively. The purpose of this study was to evaluate the robustness and reproducibility of a new sterile handheld disposable TPD to generate autologous human thrombin in the intraoperative setting, using anticoagulated whole blood as the starting source material. The performance characteristics of the thrombin were evaluated both quantitatively by measuring thrombin activity using a functional clot assay and by measuring the ability and time needed for the thrombin to complete gel formation with platelet-rich plasma.

MATERIALS AND METHODS

Collection of ACD-A Blood

Blood was drawn from healthy donors. Ethical committee and donor approval was granted from humans to draw blood for *in vitro* study. The blood was collected in 60-mL ACD-A (8%) anticoagulated syringes obtained from prescreened healthy volunteers. The blood was handled in accordance with established procedures for the collection and processing of human blood products. Each blood unit was processed within 6 hours of initiation of the collection.

Thrombin Processing Device

The TPD (Thermogenesis Corp., Rancho Cordova, CA) consists of two parts: a reaction chamber containing a negative surface charge required for initiation of the formation of thrombin and a reagent consisting of calcium chloride and ethanol at concentration of 25 mmol/L and 66%, respectively (Figure 1).

Preparation of Thrombin

Eleven milliliters of anticoagulated blood and 4 mL of thrombin reagent were added to the TPD reaction chamber. The contents were mixed and incubated for 20 minutes. The TPD was agitated to break any formed fibrinogen clots and incubated for an additional 5 minutes. After elapsed time, the TPD was shaken vigorously to dislodge the gel. The syringe was attached to the thrombin collection port, and the thrombin-activated product was collected.

To establish the intra-variability of the TPD system, the same blood was used with 10 different TPD disposables. The inter-variability was assessed by determining the thrombin activity in 25 units of thrombin produced from different blood units using TPD disposables from the same production lot. To study the influence of the ambient temperature on the production of thrombin, TPD-thrombin was prepared at 18°C and 24°C. To study the influence of ambient temperature on thrombin production, the process was performed at two different temperatures: 18 and 24°C.

Thrombin Activity and Quantitative Assessment of Thrombin

Thrombin activity was analyzed using the Clauss method as described previously (19).

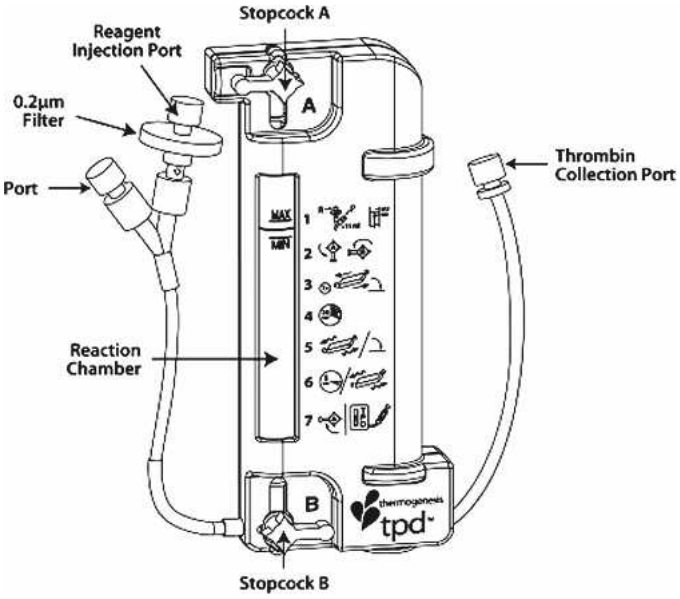

Figure 1. Description of TPD: a reaction chamber containing negatively charged particles, reagent port, and the thrombin harvesting port.

Clot Time and Qualitative Assessment of Thrombin

Platelet-rich plasma (PRP) was prepared using 60 mL ACD-A anticoagulated blood on the Magellan device as per manufacturer's instructions. One part thrombin and 10 parts of PRP (5–7× platelets over baseline and fibrinogen concentration 2–4 mg/mL) was mixed using a double lumen syringe, and the time to clot formation was assessed. The strength and elasticity of the clot were analyzed by stretching the clot. The fibrinogen component of PRP causes the formation of the clot in the presence of thrombin. To establish the relationship between clot time and thrombin activity, both analyses were performed, and a correlation was established.

Assessment of Stability

The stability of the produced thrombin was assessed by storing 3 mL of thrombin either at production temperature (i.e., 18°C or 24°C) or at 4°C. Thrombin activity was assessed at time 0 (after preparation) and after 1 and 4 hours of storage.

Statistical Analysis

All results are presented as mean and SD. Coefficient of variation (CV) was calculated for the intra- and inter-variability. A p value between groups was assessed using the Student t test.

RESULTS

Intra-Reproducibility of Thrombin Produced at 24°C

To establish the intra-variability of the thrombin production system, the same blood was used with 10 different TPDs. Using blood from the same unit and 10 different TPDs (intra-reproducibility), the thrombin activity was 40 ± 2.1 IU/mL (range, 38–43 IU/mL), with a CV of 5% (Table 1).

Inter-Reproducibility of Thrombin Produced at 24°C

Inter-variability was assessed by determining the thrombin activity in 25 TPD systems from different blood units. The inter-reproducibility was established from 10 different blood units using TPDs from the same lot of material. Thrombin activity was found to be 61 ± 12.2 IU/mL (range, 45–87 IU/mL), with a CV of 20% (Table 2).

Table 1. Thrombin production at 24°C: intra-reproducibility of thrombin produced from whole blood using TPD.

	Thrombin Harvest Volume (mL)	Thrombin Activity (IU/mL)
Mean ± SD (n = 5)	9 ± 0.7	40 ± 2.1
CV	7%	5%
Range (min–max)	8–9	38–43

Thrombin activity is expressed as mean ± SD.

Table 2. Thrombin production at 24°C: inter-reproducibility of thrombin produced from whole blood using TPD.

	Thrombin Harvest Volume (mL)	Thrombin Activity (IU/mL)
Mean ± SD (n = 20)	9 ± 0.6	61 ± 12.2
CV	7%	20%
Range (min–max)	8–10	45–87

Thrombin activity is expressed as mean ± SD.

Short-Term Stability of Thrombin Produced at 24°C

The results are shown in Table 3. For thrombin prepared at 24°C, the activity was 61 ± 12.2 IU/mL (range, 45–87 IU/mL), with a CV of 20%. When samples were stored at 4°C for 4 hours, the thrombin activity was 64 ± 15.1 IU/mL (range, 41–102 IU/mL), with a CV of 23%. The data also showed that thrombin prepared at normal ambient temperature (24°C) is sufficiently stable to effectively form fibrin clots and platelet gels when stored up to 1 hour at 24°C.

Correlation Between Thrombin Activity and Clot Time With PRP

Thrombin activity and clot times were analyzed in 18 samples. Clot formation was analyzed by mixing 1 part thrombin and 10 parts PRP. Results for thrombin activity and *in vitro* clot times with PRP using different blood units are shown in Figure 2. The total population (n = 18) with a thrombin activity of >30 IU/mL had 12 ± 1.8 seconds in clot time. Results suggest that a minimum thrombin activity of 30 IU/mL will be needed to achieve clot times ≤15 seconds.

Quality and Stability of TPD-Thrombin Produced at Different Temperatures

The ambient temperature in the operating room, where the whole blood thrombin would be produced, can vary; therefore, we assessed the quality of thrombin at 18 and 24°C. The stability of the TPD-produced thrombin was assessed by measuring its biological activity at 1 and 4 hours after production. The stability was determined at the production temperature (18 and 24°C, respectively) and at 4°C.

Stability of TPD-Thrombin Produced at 18°C

Consistent with previous findings on plasma-derived thrombin with TPD, whole blood thrombin produced at a

Table 3. Short-term stability of thrombin produced at 24°C from whole blood stored at 4°C and 24°C.

	Thrombin Activity (IU units/mL)		
	T_0	T_4 at 4°C	T_1 at 24°C
Mean ± SD (n = 20)	61 ± 12.2	64 ± 15.1	63 ± 14.6
CV	20%	31%	23%
Range (min–max)	45–87	41–102	41–99

Thrombin activity is expressed as mean ± SD.

Table 4. Stability of thrombin produced by the TPD system: thrombin was produced at 18°C from whole blood and stored for 1 and 4 hours at 4°C and 18°C.

	Thrombin Activity (IU units/mL)		
n = 25	After Harvest	After 4 Hours	After 1 Hour
Storage	NA	4°C	18°C
Mean	52 ± 13.9	43 ± 12.2	38 ± 12.4
CV	27%	29%	33%
Range (min–max)	31–81	25–73	16–70

Thrombin activity is expressed as mean ± SD.

Table 5. Stability of thrombin produced by the TPD system: thrombin was produced 24°C from whole blood and stored for 1 and 4 hours at 4°C and 24°C.

	Thrombin Activity (IU units/mL)		
n = 25	After Harvest	After 4 Hours	After 1 Hour
Storage	NA	4°C	24°C
Mean	61 ± 12.2	64 ± 15.1	63 ± 14.6
CV	20%	23%	23%
Range (min–max)	45–87	41–102	47–99

Thrombin activity is expressed as mean ± SD.

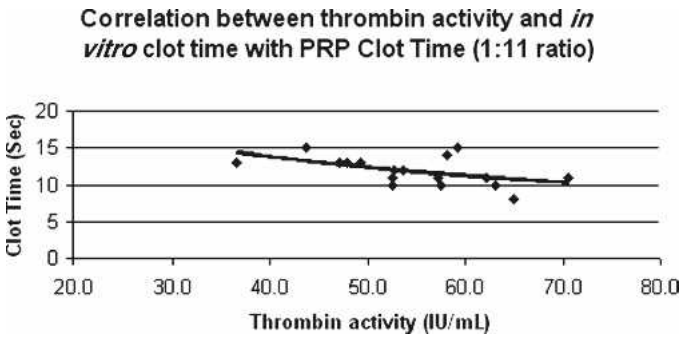

Figure 2. Correlation between thrombin activity and *in vitro* clot times with PRP (1 part thrombin + 10 parts PRP) using different blood units.

lower ambient temperature (18°C) did not retain its thrombin activity as well as thrombin produced at higher ambient temperatures. After 4 hours of storage at 4°C, the thrombin activity had decreased to 43 ± 12.2 IU/mL (25–73 IU/mL). After 1 hour of storage at 18°C, we found that the thrombin activity decreased to 38 ± 12.4 IU/mL (range, 16–70 IU/mL; Table 4).

Stability of TPD-Thrombin Produced at 24°C

Thrombin prepared and stored at normal ambient temperature (24°C) showed no significant difference in observed thrombin activity (61 ± 12.2 vs. 63 ± 14.6 IU/mL) after 1 hour (Table 5). Similarly, when the same thrombin was stored at 4°C for 4 hours, no significant difference in thrombin activity was observed. The data suggest that thrombin prepared at normal ambient temperature (24°C) retained clotting activity for 1 hour at room temperature (18–26°C) and up to 4 hours when stored on ice.

DISCUSSION

The results of this study confirm our "proof of concept" data published in 2007 (20) that a practical process for the routine production of human thrombin from autologous whole blood in the intraoperative setting can be achieved. The whole blood technique we developed is derived from the method that we have previously commercialized using single donor plasma. Currently, thrombin used in surgical procedures as an adjunct to hemostasis (3–11) is primarily derived from bovine sources and is associated with major concerns about adverse reactions, e.g., antibody formation against human factor V leading to bleeding episodes (12,13), and transmission of bovine prions possibly causing vCJD (14). An alternative choice of using autologous thrombin is an attractive alternative choice in surgeries because it avoids risk of infectious disease and immunogenicity issues. This whole blood TPD procedure provides a more convenient and faster method than our previous plasma procedure because it eliminates the need for centrifugation for preparing the plasma. The entire procedure can readily be performed in <30 minutes. For this study, freshly drawn blood from normal tested donors was used within 8 hours of collection. In surgery, the blood will be taken directly from the patient, and thrombin will be produced intraoperatively.

The enzymatic activity of thrombin to be used either by itself or in combination with fibrinogen or platelets is important. PRP contains a high concentration of platelets, which can be activated by thrombin to form platelet gel, followed by release of growth factors [platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β)] for therapeutic use. These growth factors released from activated platelets participate in the wound healing process. The thrombin produced in this study ranged in activity from 31 to 87 IU/mL, with a mean of 52 IU/mL. This thrombin activity is very similar to our previous observation with plasma using TPD (19). In addition, we have previously shown that TPD-produced thrombin can activate platelets to release α-granule content, and consequently growth factors, comparable to commercially available thrombin (21).

When 10 units of TPD-thrombin were produced from the same unit of blood, the CV was 12%, indicating the ability of the TPD to produce thrombin with a consistent thrombin activity. Furthermore, when blood from different donors was used, the CV increased to 25%. Thus, there is variability between donors/blood in the ability to produce active thrombin using the TPD. This would be expected because availability of prothrombin and other coagulation factors needed for formation of the prothrombinase complex can vary between donor bloods. The results suggest that the TPD is able to produce a relatively consistent thrombin with acceptable activity.

Using *in vitro* clot time with PRP as a qualitative mea-

sure of the efficacy of the thrombin depends on the ratio of thrombin to PRP. In most cases, a clot time of 10–15 seconds was equal to instant clotting, because of the manual variability of the test. Data from this study showed that clot times with platelet concentrates at 1:11 ratio were <15 seconds. This *in vitro* clot time suggests that lower levels of thrombin activity than what is commonly used today (500–1000 IU/mL) would be sufficient for adequate clot formation in vivo. In fact, using a combination of thrombin and fibrinogen in a fibrin sealant, it has been shown that a thrombin with an activity of 50–100 IU/mL had a higher adhesiveness compared with one with a thrombin activity of 500 IU/mL (22).

Typically, enzymes are sensitive to temperature, and the rate of activation and degradation is dependent on the specific optimal temperature for each enzyme. Our data suggested that thrombin produced at 24°C had a higher activity and retained its activity for a longer time compared with thrombin produced at a lower temperature (18°C). This observation is very important for the user. Because thrombin is an active enzyme, the user has to be aware of both the production temperature (the ambient temperature in the room) and the storage temperature for optimal efficacy of the product. For example, if the ambient temperature is 24°C, the TPD-thrombin can be stored for up to 1 hour without any special precautions (i.e., it can be stored on the bench). If longer storage time is needed, the thrombin should be stored at 4°C. However, if the ambient temperature is in the range of 16–18°C, as is the case in certain surgical procedures, the shelf-life of the thrombin is shorter, and it should be used within 1 hour of production or stored on ice.

In conclusion, we showed that it is practical to produce active and effective thrombin from whole blood at low and normal ambient temperatures. This study showed that the activity and *in vitro* clot times of thrombin are dependent on both production and storage temperatures. Thrombin produced at 18 and 24°C retained clotting efficacy for 4 hours when stored at 4°C. Thus, we developed a simple, rapid, and reliable method for generation of stable thrombin from patient blood in ~30 minutes.

REFERENCES

1. Mann KG. Thrombin formation. Chest. 2003;124(3 Suppl):4S–10S.
2. Aziz O, Athanasiou T, Darzi A. Haemostasis using a ready-to-use collagen sponge coated with activated thrombin and fibrinogen. Surg Technol Int. 2005;14:35–40.
3. Hashemi K, Donaldson LJ, Freeman JW, Sokhi GS, Gyde OH, Smith HV. The use of topical thrombin to reduce wound haematoma in patients receiving low-dose heparin. Curr Med Res Opin. 1981;7: 458–62.
4. Suzuki R, Matsuhashi M, Kuwahara T, Kase T. Topical use of thrombin in prostatic surgery. Hinyokika Kiyo. 1986;32:1461–64.
5. Ofodile FA, Sadana MK. The role of topical thrombin in skin grafting. J Natl Med Assoc. 1991;83:416–8.
6. Sarpel U, Roayaie S, Schwartz ME, Labow DM. The role of fibrin sealants in hepatic surgery. Surg Technol Int. 2007;16:31–6.
7. Franz RC. Platelet concentrate-thrombin coagulum: a new biological dressing for the promotion of wound healing. S Afr Med J. 1987;72: 810–1.
8. Amrani DL, Diorio JP, Delmotte Y. Wound healing. Role of commercial fibrin sealants. Ann N Y Acad Sci. 2001;936:566–79.
9. Moore M, Burak W Jr, Nelson E, et al. Fibrin sealant reduces the duration and amount of fluid drainage after axillary dissection: a randomized prospective clinical trial. J Am Coll Surg. 2001;192: 591–9.
10. Slater M, Patava J, Kingham K, Mason RS. Involvement of platelets in stimulating osteogenic activity. J Orthop Res. 1995;13:655–63.
11. Anitua E, Andia I, Ardanza B, Nurden P, Nurden AT. Autologous platelets as a source of proteins for healing and tissue regeneration. Thromb Haemost. 2004;91:4–15.
12. Crow SS, Sullivan VV, Aysola AE, et al. Postoperative coagulopathy in a pediatric patient after exposure to bovine topical thrombin. Ann Thorac Surg. 2007;83:1547–9.
13. Neschis DG, Heyman MR, Cheanvechai V, Benjamin ME, Flinn WR. Coagulopathy as a result of factor V inhibitor after exposure to bovine topical thrombin. J Vasc Surg. 2002;35:400–2.
14. Beghi E, Gandolfo C, Ferrarese C, et al. Bovine spongiform encephalopathy and Creutzfeldt-Jacob disease: Fact and uncertainties underlying the causal link between animal and human disease. Neurol Sci. 2004;25:122–9.
15. Cohn E, Oncley J, Strong L, Hughes W Jr, Armstrong H Jr. Chemical, clinical, and immunological studies on the products of human plasma fractionation I. The characterization of the protein fractions of human plasma. J Clin Invest. 1944;23:417–32.
16. Fenton J 2nd, Fasco M, Stackrow A. Human thrombins. Production, evaluation, and properties of alpha-thrombin. J Biol Chem. 1977;252: 3587–98.
17. Seegers W. Activation of purified prothrombin. Proc Soc Exp Biol Med. 1949;72:677–80.
18. Stocker K, Hauer H, Muller C, Triplett DA. Isolation and characterization of Textarin, a prothrombin activator from eastern brown snake (*Pseudonaja textilis*) venom. Toxicon. 1994;32:1227–36.
19. Kumar V, Madsen T, Zhu H, Semple E. Stability of human thrombin produced from 11 ml of plasma using the Thrombin Processing Device—TPD™. J Extra Corpor Technol. 2005;37:390–5.
20. Kumar V, Chapman JR. Whole blood thrombin: development of a process for intra-operative production of human thrombin. J Extra Corpor Technol. 2007;39:18–23.
21. Semple E, Madsen T, Semple J. Quality of thrombin produced from the patient's own plasma using the TPD™, a new thrombin processing device. J Extra Corpor Technol. 2005;37:196–200.
22. Yoshida H, Hirozane K, Kamiya A. Adhesive strength of autologous fibrin glue. Biol Pharm Bull. 2000;23:313–7.