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# An Ethanol-Free Autologous Thrombin System

Andrea M. Matuska, PhD;* Marina K. Klimovich, BS;† John R. Chapman, PhD†

*Arthrex, Inc., Naples, Florida; and †Stem Cell Partners, LLC, Sacramento, California

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**Abstract:** Thrombin is a coagulation protein of central importance to hemostasis and wound healing that can be sourced from human blood, bovine blood, and engineered cell lines. Only autologous thrombin lacks the risks of transmitting emergent pathogens or eliciting an immunogenic response. Previous commercial autologous thrombin devices require the use of high concentrations of ethanol to achieve thrombin stability, introducing cytotoxicity risks. A new point of care device for preparing an ethanol-free autologous thrombin serum was investigated. The ethanol-free autologous serum (AS) was prepared using the Thrombinator™ System (Arthrex, Inc., Naples, FL). A total of 120 devices were tested with the blood of 30 healthy donors to determine the reliability and flexibility of the procedure. AS was prepared from both whole blood (WB) and platelet-poor plasma (PPP). Study endpoints were thrombin activity determined using a coagulation analyzer and formation of cohesive bone graft composites objectively measured using a durometer. The average thrombin activity produced by this system from 24 donors was 20.6 ± 2.7 IU/mL for WB and 13.4 ± 3.8 IU/mL for PPP which correlated to clot times of 3.9 and 5.9 seconds, respectively. The device tolerated use of varying volumes of blood to prepare AS. In addition, the system was able to generate four successive and comparable AS productions. When combined with platelet-rich plasma and bone graft material, cohesive scaffolds were always formed. A new device and method for preparing single donor, ethanol-free, AS with thrombin activity was demonstrated. **Keywords:** thrombin, autologous, blood-coagulation, platelet rich plasma, fibrin, device. *J Extra Corpor Technol. 2018;50:237–43*

Thrombin plays an important role in the body's response to injury. Thrombin acts as an agonist causing platelet activation and aggregation via activation of protease-activated receptors on the cell membrane of the platelet. Platelets contain more than 1,100 proteins including growth factors, immunomodulatory factors, enzymes, and inhibitors. These factors can improve tissue repair by diverse mechanisms including regulation of inflammation, angiogenesis, synthesis, and remodeling of new tissues (1–3). Activation of platelets causes the release of these factors, and platelet-rich plasma (PRP) activated with thrombin has been used in various fields with promising results such as odontology, plastic surgery, orthopedics, wound healing, and aesthetics (4–8).

Thrombin also acts as a serine protease that catalyzes the conversion of soluble fibrinogen into fibrin monomers. In the presence of thrombin and calcium, factor XIII becomes activated and catalyzes the formation of cross-links in fibrin to form a stable clot (9,10). When platelet concentrates and surgical grafting materials are combined with thrombin and calcium, the formed fibrin matrix facilitates handling and acts as a scaffold for cell and tissue ingrowth (11).

Thrombin-based clotting agents currently used for topical hemostasis, fibrin sealants, and platelet gels are primarily derived from bovine blood, Chinese hamster ovary cell line using recombinant technology, or pooled human plasma prepared from many donors. Autologous thrombin has important safety advantages in that it does not carry the pathogen safety concerns as pooled plasma–derived products, and it avoids exposure to animal-derived recombinant deoxyribonucleic acid (DNA) proteins with attendant immunogenicity risks. All efforts toward a commercial medical

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Address correspondence to: Andrea M. Matuska, PhD, Research Scientist, Department of Research and Development, Arthrex, Inc., 1370 Creekside Boulevard, Naples, FL 34108. E-mail: andrea.matuska@arthrex.com
Andrea Matuska is an employee of Arthrex, Inc., and Marina Klimovich and John Chapman are contracted consultants of Arthrex, Inc., Naples, FL.



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device for the preparation of autologous thrombin have relied on the use of high concentrations of ethanol (>15%) to interfere with the binding of anti-thrombin III to thrombin as taught by Sternberger in 1947 (12). Instead of fully achieving the safety benefits offered by autologous blood, these systems have introduced a new safety concern, which is ethanol-mediated cytotoxicity on the blood fluid and body tissue at the point of contact during application.

The technical challenge for autologous sources of thrombin is not the production of robust amounts of thrombin, but rather in having thrombin remain reliably present in an active form for a sufficient time to be feasible for clinical use. The present study investigates a new medical device, the Thrombinator™ System (Arthrex, Inc.). The basic premise relies on the prothrombinase enzyme complex which catalyzes the conversion of prothrombin (Factor II), an inactive zymogen, to thrombin (Factor IIa) (13). As demonstrated herein, the prothrombinase complex, once formed, remains functionally active for at least 4 hours which enables an alternative approach for creating an autologous serum (AS) that does depend on the use of ethanol. The aim of this study was to investigate the reliability, stability, and flexibility of the Thrombinator System for preparing AS.

## MATERIALS AND METHODS

### Preparation of Platelet-Poor Plasma (PPP) and PRP

Fresh whole blood (WB) from adult, pre-screened, normal, healthy volunteers was purchased from a commercial source (Stem Express, Folsom, CA). Blood was collected, with informed consent and Institutional Review Board (IRB) approval (WIRB-Copernicus Group, Princeton, NJ, IRB No. 20152875), containing 13% (v/v) of anticoagulant citrate dextrose-solution A (ACD-A). Blood was processed and complete testing was performed within 8 hours of phlebotomy. PRP and PPP were prepared using the Arthrex Angel® cPRP System (Naples, FL) as per manufacturer's instructions. Cell counts were measured using a hematology analyzer (Horiba Micro60 Analyzer Model M60CS, Horiba Medical, Irvine, CA). The PRP had an average concentration factor of 6.8× the initial platelet count and an average hematocrit of 6%. The PPP had a four-fold reduction from the initial platelet count and no detectable hematocrit. The blood was handled and disposed of according to established procedures for the collection and processing of human blood products.

### Quantitative Assessment of Clotting Activity of AS

Clotting activity was analyzed using the Diagnostica Stago STart® 4 Hemostasis Analyzer (Parsippany, NJ). The device is a semi-automated benchtop system that uses an electromechanical clot detection method based on viscosity with automated timers. Briefly, 100 μL of pre-warmed (37°C) pooled plasma (Interstate Blood Bank, Memphis, TN) was added to 100 μL of the AS in a cuvette. The time required for clot formation was automatically detected and recorded by the device, with testing of each sample repeated in quadruplicate and averaged to produce a clot time measured in seconds for that sample. A standard curve was constructed using serial dilutions of a commercially available thrombin reagent (Fibrinogen V Thrombin Standard) having 83 IU/mL when reconstituted per manufacturer's instructions (Diagnostica Stago). Because each clot time point (seconds) on the standard curve corresponds to the thrombin activity needed to clot a standardized concentration of fibrinogen; the thrombin activity in an unknown sample can be determined by the time to clot formation.

### Production of AS Using the Thrombinator™ System-General Method and Variations

The Arthrex Thrombinator™ System consists of a gamma-sterilized tube made of medical grade plastic containing a controlled amount of negatively charged borosilicate glass beads and a permanently sealed lid containing a vent and two needleless swabbable valves (Figure 1). One valve is marked for injection of fluids into the device, and one valve is marked for extraction of fluids. A dedicated syringe filter with a 17-micron pore size for extracting the thrombin serum from the device is provided with the system. The Thrombinator System can produce AS with either WB or PPP.

Briefly, the principal of operation of the Thrombinator System consists of first activating the device by the addition of an initial recalcified blood fluid aliquot to generate the

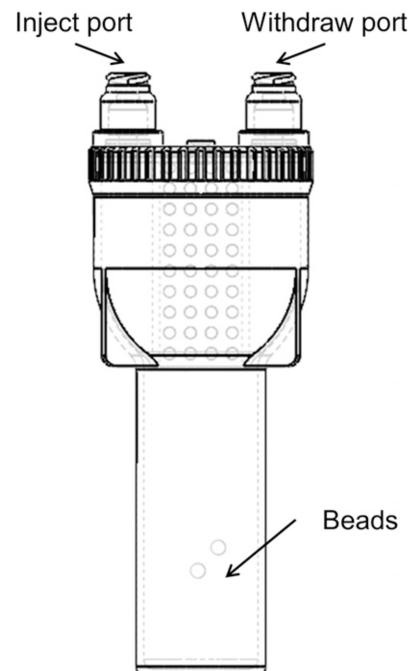


**Figure 1.** Schematic of the device demonstrating inject and withdraw ports and location of the borosilicate beads.

stable prothrombinase complex. The activated device is stored at room temperature on the bench until the approximate time AS is anticipated to be needed. The AS can then be generated within 2 minutes by injecting a second blood fluid aliquot into the device and harvesting the fluid contents out of the device. The mechanism of action for the two-step procedure is that the first aliquot initiates the entire coagulation cascade including building the stable prothrombinase enzyme complex, and the second aliquot provides fresh prothrombin for rapid enzymatic conversion to thrombin by the prothrombinase enzyme complex. In this study, 10% calcium chloride ($CaCl_2$, 1.36 mEq, International Medication Systems, South El Monte, CA) was used for recalcification of ACD-A anticoagulated blood. Room temperature was controlled to be between 17 and 21°C. The same blood fluid was used for both the production step and activation step.

Various parameters within the device procedure were evaluated in this study. The activation step always consisted of adding .1 mL of 10% $CaCl_2$ and 4 mL of blood fluid (ACD-A anticoagulated WB or PPP) to the device. After mixing the contents of the device by brief shaking, the device was laid horizontally on the laboratory bench and allowed to incubate undisturbed at room temperature until clotted. To assess the stability of the prothrombinase complex at room temperature, varying time points from 0 to 4 hours were evaluated at this step before proceeding. Unless otherwise stated, the production step was 8 mL blood fluid; however, the use of 4 or 12 mL of blood fluid for the production step was also evaluated. Recalcification of the blood fluid was always achieved by adding .1 mL of 10% calcium chloride per 4 mL of blood fluid. All AS was harvested via the extraction port through the included 17 micron filter into a 20 mL syringe. During storage studies, AS was stored on wet ice. Experiments were designed to characterize the in vitro performance characteristics of the Thrombinator System (Table 1).

### Quantitative and Qualitative Assessment of AS for Preparation of Bone Grafts

Functional testing of the AS was performed by preparing bone graft composites. Graft composites were made with demineralized cortical allograft bone (CellRight Technologies, Universal City, TX). The allograft material (.5 g) was hydrated in the barrel of a 10 mL syringe with 2.5 mL of PRP. This composite was quickly mixed with 1 mL of AS and incubated undisturbed for 5 minutes. The graft was removed from the syringe, placed in a petri dish, and gently blotted to remove excess water. The resistance of the graft to permanent indentation was measured using a Model DD-4 Type 000 Durometer with 0S-4H operating stand (Rex Gauge, Buffalo, IL), which measures the graft hardness on a scale of 0 (extremely soft) to 94 (extremely hard). After durometer testing, each graft was picked up with a gloved hand to determine if the intended improved handling characteristics had been qualitatively achieved. The endpoint of the handling test was pass/fail for the graft being able to be lifted without tearing. AS prepared using both WB and PPP were tested.

### Statistical Analysis

All results are presented as mean and SD. When data were normally distributed a one-way or repeated measure analysis of variance (ANOVA) was used to determine significant difference between groups ($\alpha = .05$). When significance was noted, post hoc Tukey's tests were used to determine significant difference between individual groups.

## RESULTS

### Reliability of the Procedure

Twenty-four units of blood anti-coagulated with ACD-A (13% v/v) from 13 females and 11 males with an age range of 18–67 were tested (Figure 2). The Thrombinator System was evaluated with both WB and PPP by generating AS either immediately after device activation (0 Hour Device, T0) or by activating a device and then waiting for 4 hours before generating AS (4-Hour Device, T4D). On average, device activation times recorded before proceeding were 14.8 ± 2.0 and 18.2 ± 3.1 minutes for WB and PPP devices, respectively. Devices were allowed to fully gel before proceeding for consistency, however partial gels still indicate that the device is in an active state and this occurred 2–4 minutes before the recorded times. Thrombin activity was determined immediately after AS harvest. In addition, the AS produced from the 0 hour device was stored for

**Table 1.** Summary of experiments performed to characterize the Thrombinator System.

| Experiment | Blood Donors | Devices Used | Step 1: Activation | Device Storage (hour) | Step 2: Production | Serum Storage (hour) |
|---|---|---|---|---|---|---|
| Reliability of the procedure | 24 | 96 | 4 mL (WB or PPP) | 0 or 4 | 8 mL (WB or PPP) | 0 or 4 |
| On demand use and storage profiles | 3 | 18 | 4 mL (WB or PPP) | 0, 2 or 4 | 8 mL (WB or PPP) | 0–4 |
| Flexibility of production volume | 12 | 72 | 4 mL (WB or PPP) | 0 | 4, 8, or 12 mL (WB or PPP) | 0 |
| Additional AS production | 3 | 6 | 4 mL (WB or PPP) | 0 | 8 mL (WB or PPP) repeated up to 4× | 0 |
| Functional testing: Bone graft preparation | 12 | 48 | 4 mL (WB or PPP) | 0 or 4 | 8 mL (WB or PPP) | 0 or 4 |

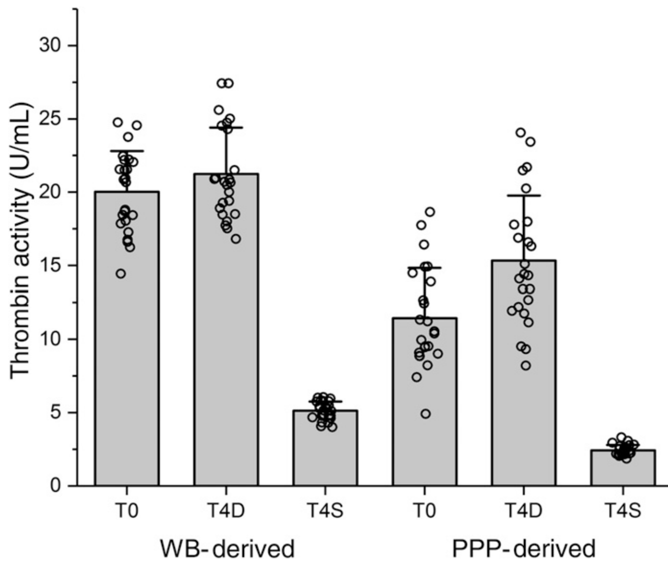


**Figure 2.** Thrombin activity (U/mL) measured in AS generated from either WB or PPP from 24 different donors. T0-AS produced immediately after activation, T4D-AS produced 4 hours after activation, T4S-AS stored 4 hours on ice.

4 hours on ice and thrombin activity determined after storage (T4S). For WB, no difference ($p$ = .468) in thrombin activity was observed for AS produced immediately after activation as compared with AS produced in devices that were stored for 4 hours after activation. For plasma, a difference in thrombin activity was observed for AS produced immediately after activation compared with AS produced in devices stored for 4 hours after activation ($p < .001$), where 4-hour–stored devices showed greater thrombin activity. AS generated from WB had higher thrombin activity compared with PPP ($p < .001$). AS prepared from devices stored for 4 hours after activation had higher thrombin activity compared with AS which had been stored on ice for 4 hours (T4S). This finding supports the concept of first activating the Thrombinator device and then generating the AS proximate to the time of its intended use. Average clotting times for WB- and PPP-derived AS were 3.9 and 5.9 seconds, respectively. When using a total input volume of 12 mL (4 + 8 mL) into the device, overall volume of AS harvested was 7.0 ± .5 mL when using WB and 6.9 ± .7 mL when using PPP.

### On-Demand Use and Storage Profiles

The ability of using stored Thrombinator devices for on-demand thrombin production and thrombin activity persistence more than 4 hours was evaluated (Figure 3). There was no negative effect on AS production when activated Thrombinator devices were stored mid-production for either 2 or 4 hours. As stated before, thrombin activity was greater in AS produced using WB as compared with PPP. Generated AS had similar storage profiles regardless of

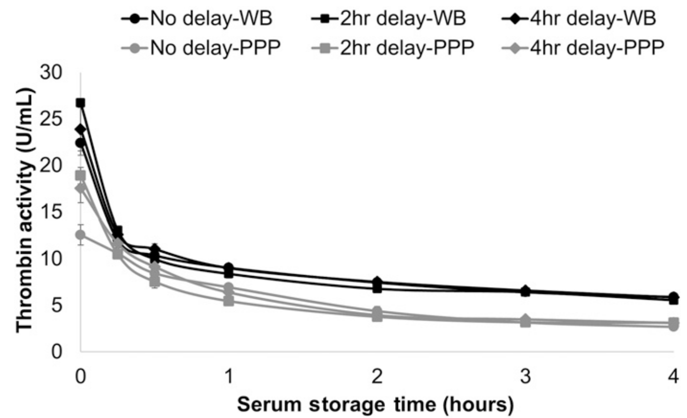


**Figure 3.** Device storage for on-demand thrombin and thrombin activity storage profile (n = 3).

mid-procedure storage. The level of thrombin activity was greater than 5 IU/mL for all WB samples even after 4 hours of storage on ice. The level of thrombin activity was greater than 5 IU/mL for all AS prepared using PPP for up to 1 hour of storage and remained greater than 2.5 IU/mL up to 4 hours of storage on ice.

### Flexibility of Production Volume

The device can accept from 4 to 12 mL of blood fluid during the production step without overtly affecting the thrombin activity of the AS produced (Figure 4). In fact, increasing volume additions with WB (8 and 12 mL) resulted in a significant increase in thrombin activity compared with 4 mL ($p$ = .030 and $p$ = .006, respectively), whereas there was an inverse effect with PPP ($p < .001$, $p$ = .006, respectively). Although any differences are likely not functionally relevant as they corresponded to average clot times of 4.5, 4.1, and 4.0 seconds for WB-derived AS and 6.1, 7.2, and 7.8 seconds for PPP-derived AS. The volume of AS recovered was proportional to the volume of blood fluid input into the Thrombinator device.

### Additional AS Production

Thrombin activity in AS was measured immediately after harvest from four successive production steps (Figure 5). There was no statistically significant increased or decreased thrombin activity with successive AS yields for either WB or PPP blood fluids ($p$ = .055 and $p$ = .635, respectively). Successive WB-derived AS productions had 20.9 ± 2.2, 25.2 ± 1.4, 22.9 ± 2.2, and 21.5 ± 2.2 U/mL of thrombin activity where PPP-derived AS productions had 10.1 ± 1.3, 9.7 ± 1.1, 9.3 ± .3, and 8.9 ± .2 U/mL thrombin activity. The filter remained functional over four extractions of AS from the Thrombinator device. As previously observed, there was greater thrombin activity in AS prepared using WB compared with PPP.

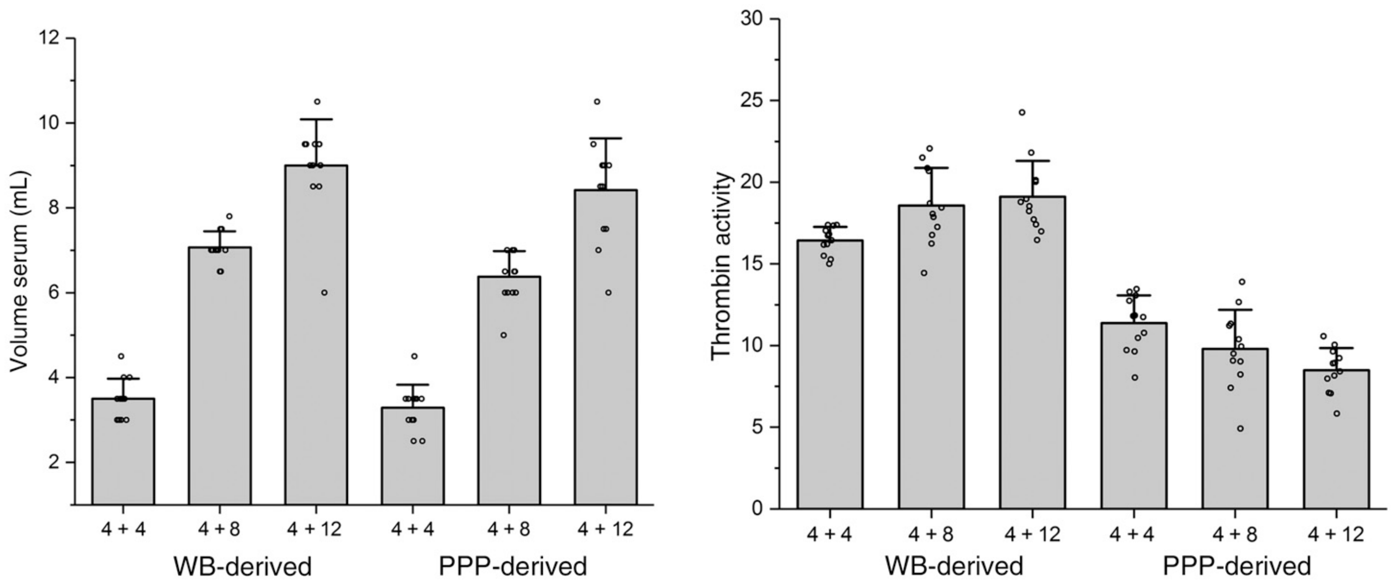


**Figure 4.** Effect of different volume additions in the second step on final AS yield and thrombin activity (n = 12). 4 + 4- 4 mL production step, 4 + 8- 8 mL production step, 4 + 12- 12 mL production step.

**Bone Graft Composites Prepared Using AS, PRP, and Demineralized Bone Matrix**

The AS used for this study were from 12 of the donors shown in Figure 1 (demonstrated thrombin activity). A total of 72 grafts were consecutively prepared, and all graft preparations were observed to produce cohesive bone grafts that could be readily picked up and handled without tearing. The data generated using the durometer indicated that grafts prepared using PPP were more resistant to deformation than grafts prepared using WB (Figure 6). AS that had been stored for 4 hours on ice still had sufficient thrombin activity to create functional grafts. The necessity for using AS for graft production was demonstrated by substituting it with PPP and observing that the graft remained in liquid form, effectively resulting in a zero durometer reading (data not shown).

## DISCUSSION

The primary objective of this study was to characterize the thrombin activity of AS prepared using the Thrombinator

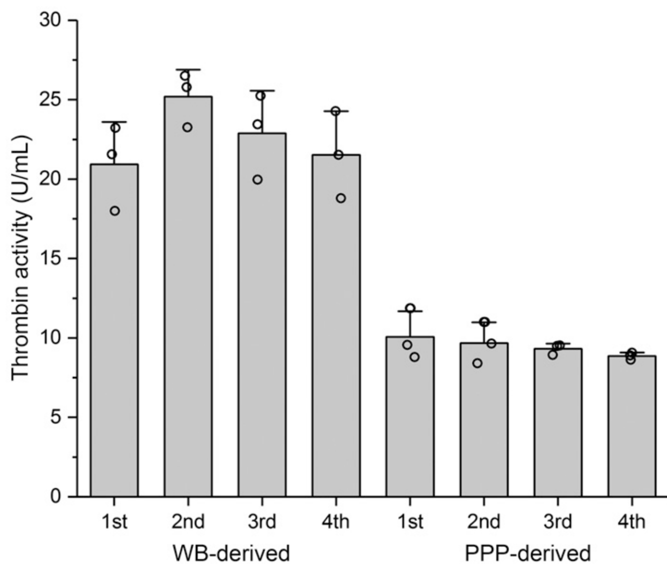


**Figure 5.** Additional AS production up to four times showed no effect on thrombin production (n = 3).

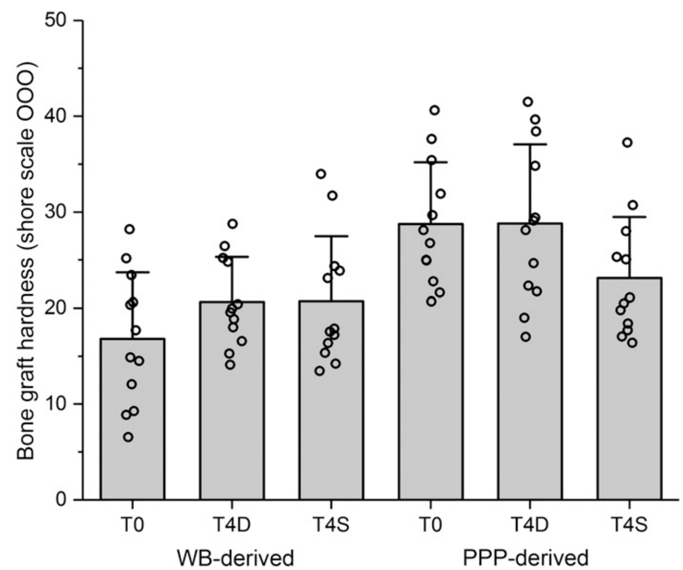


**Figure 6.** Functional testing of WB- or PPP-derived AS using bone graft hardness tested by a durometer (n = 12). T0-AS produced immediately after activation, T4D-AS produced 4 hours after activation, and T4S-AS stored 4 hours on ice.

System. A total of 120 devices were tested with the blood of 30 healthy donors without any failures. Thrombin activity of the AS was found to have a SD that was less than 15% of the mean for WB and less than 30% of the mean for PPP regardless if the production step was performed immediately after activation or after a delay of 4 hours. The system exhibited flexibility to use different volumes of blood fluid during the production step without compromising the thrombin activity of the resultant AS. In addition, the system was able to generate four successive and comparable AS productions within a single device using the same filter and blood source.

Generated AS had between 10 and 30 IU/mL of thrombin activity, depending on the input fluid, and a minimum of 5 IU/mL. Interestingly, a consistent finding of the study was that higher thrombin concentrations were observed in the AS produced using WB as compared with PPP. With prothrombin naturally circulating within plasma, platelets play a further role in activation of prothrombin by supplying thromboplastin and other factors which can increase the speed of prothrombin conversion (13,14). Therefore, this observed difference may be partially due to the reduction in platelets in PPP compared with WB; however, further work would be needed to confirm this hypothesis. Although not tested in this study, it would also be possible to perform the two separate steps using different blood fluids from the same donor (i.e., WB and then PPP).

The concentrations generated within this system are much lower than commercially available purified preparations (human or bovine) that have concentrations of thrombin from 100 to 1,000 U/mL. However, the concentrations that were produced are at a more physiological magnitude which may offer additional benefits such as natural clot structure and maximum platelet activation, which occurs above 1 U/mL (15). As demonstrated in controlled additive studies by Ryan et al. the time to clot formation greatly decreased at more than 1 U/mL even though low concentrations (<1 U/mL) are sufficient to induce clotting (16). Lower thrombin concentrations generally result in thick loosely woven fibrin strands, whereas higher concentrations produce clots that comprise thinner tightly packed strands (17). In vitro studies have demonstrated that thrombin and fibrinogen concentrations plays a role in fibroblast and mesenchymal stem cell (MSC) morphology, proliferation, and migration within fibrin clots where supra-physiologic levels of each tend to have a negative effect on these outcomes (18,19). In vivo studies have also supported this notion where Gugerell et al. (20), reported that in a rat excisional wound healing model, more rapid wound closure and less wound severity was seen in wounds treated with a fibrin matrix generated from thrombin used at 4 IU/mL instead of 820 IU/mL.

When combined with demineralized cortical bone allograft, Thrombinator AS and Angel PRP were observed to provide consistently strong, cohesive bone graft composites. Interestingly, thrombin concentration did not necessarily correlate with graft hardness. Grafts prepared using PPP-derived AS were more mechanically resistant to indentation than WB-derived AS grafts. This is possibly because of the fact that increasing RBC concentrations has been shown to disrupt fibrin network formation, affecting clot mechanical strength (21).

In addition to preparing bone grafts as described previously, thrombin sourced from autologous blood or bovine sources is used for regenerative medicine applications such as activating platelets to release growth factors, platelet gels, fibrin glues, and for cell delivery within fibrin scaffolds (22). It is important, therefore, to consider the safety profiles of all approaches. Although topical bovine thrombin preparations have been widely used for hemostatic purposes in clinical practice, there is concern that exposure to bovine thrombin can result in the development of cross-reactive antibodies, usually against factor V/Va, which can lead to hemostatic abnormalities (23). Bovine thrombin has a well-known risk of immunogenicity, and since 1988, several reports have been published of patients developing anti-thrombin antibodies after being exposed to topical bovine thrombin during surgical procedures (24). The choice of using autologous thrombin, therefore, seems very attractive because there is no exposure to foreign proteins or risk of transmitting viral pathogens.

Previous attempts to have commercial thrombin devices all produce ethanol-containing autologous serum (EAS). The use of ethanol to block the inhibitory action of anti-thrombin III on thrombin has been known for more than 70 years (12). The concentration of ethanol in EAS is selected to be sufficiently low for all of the needed coagulation cascade enzymatic reactions to proceed to the point of generating thrombin but also, sufficiently high to be inhibitory to anti-thrombin III neutralizing thrombin activity. These constraints create the requirement that ethanol be used in the range of 13–25% v/v which is not biocompatible. Specific concerns are the direct ethanol mediated cytotoxicity effects on the cells present in the blood fluid being used to generate the thrombin serum, and for the cells and tissues that directly contact the EAS on use. The ability of ethanol to impair growth factor release from platelets is an additional risk factor (25). With only the addition of calcium chloride to recalcify anticoagulated blood, there are none of these concerns with an ethanol-free system. It should be noted that other anticoagulants that are not reversed with the addition of calcium, such as heparin, cannot be used.

In summary, the Thrombinator System is a new point of care medical device for the rapid point of care production of AS with only the addition of calcium chloride to recalcify anticoagulated blood. In this study, product performance was reliable, reproducible, and robust. The

ability to rapidly produce AS and store the activated device without the use of ethanol was demonstrated, and future work will evaluate the safety benefits of an ethanol-free serum.

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