

Evaluation of Platelet Gel Characteristics Using Thrombin Produced by the Thrombin Processing Device: A Comparative Study

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Purpose: Autologous platelet gels can be prepared using the patient's own platelet-rich plasma and thrombin produced by the Thrombin Processing Device (Thermogenesis Corp, Rancho Cordova, CA). As the Thrombin Processing Device thrombin contains a residual amount of ethanol, the purpose of this study was to investigate the effect of the Thrombin Processing Device thrombin on growth factor release from platelet gels, and the effect on cell viability and cell proliferation.

Materials and Methods: Platelet gels were prepared using Thrombin Processing Device-produced human thrombin at platelet-rich plasma to thrombin ratios of 3.3 to 1 and 7 to 1. Commercially available bovine thrombin was used as control. The content of the growth factors, platelet-derived growth factor beta polypeptide, and transforming growth factor beta, were assessed in both the clot and supernatant. The influence of different concentrations of ethanol on cell viability was assessed by flow cytometry and cell proliferation was assessed by ³H-thymidine incorporation.

Results: Using a ratio of 3.3 to 1, the supernatant of the platelet gel produced with Thrombin Processing Device thrombin had a lower growth factor content compared with bovine thrombin but was similar when prepared using a ratio of platelet-rich plasma to thrombin of 7 to 1. Supernatants from the platelet gels did not affect the viability of human macrophage line cells or a fibroblast cell line. When the different platelet gels or their supernatants were tested for their ability to stimulate cell proliferation, similar rates of proliferation were observed.

Conclusions: These data suggest that residual ethanol in the Thrombin Processing Device-produced thrombin does not affect any of the tested parameters and has similar characteristics as commercially available bovine thrombin.

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The use of platelet gels to enhance wound healing and bone regeneration in surgical procedures is widespread.¹⁻¹⁰ The enhancement found in wound healing and osteogenesis is explained by the release of growth factors from the platelets in the platelet gel.¹¹⁻¹²

A platelet gel is typically produced by preparing platelet-rich plasma (PRP), containing concentrated platelets and white cells, from the patient's own blood. The PRP is then combined with commercially available thrombin (bovine or human) and forms a gel that can be applied on the tissue surface to promote

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wound healing. During the formation of the gel, ie, clotting, the platelets are activated and will release growth factors such as platelet-derived growth factor beta polypeptide (PDGF-B) and transforming growth factor- β (TGF- β) from their α -granules.¹³ The amount of growth factor released is dependent on the rate of activation and the number of platelets.¹⁴⁻¹⁶

Thrombin used for activation of the PRP has to date primarily been from bovine origin. The use of bovine thrombin (BT) has caused major concern as it has been shown to cause formation of antibodies that cross-react with the patient's coagulation factor V.^{17,18} This may lead to a range of adverse reactions, including severe and life-threatening bleeding. In addition, there is a concern of possible exposure to bovine-derived prions causing variant Creutzfeldt-Jacob disease in humans.¹⁹ Thus, the ability to produce a human, preferably autologous, thrombin for surgical use has been highly desirable.

Thrombin can be readily produced from citrated plasma by adding calcium ions, thus reversing the inhibition of the coagulation caused by the citrate. However, thrombin produced by this method is not practical for clinical use because its activity is typically decreased to nonfunctional levels within 20 minutes.²⁰ In order to circumvent these limitations, a simple method to produce stable thrombin was developed (the Thrombin Processing Device [TPD; Thermogenesis Corp, Rancho Cordova, CA]) where a mixture of calcium chloride and ethanol provides the chemical constituents needed to activate thrombin, inactivate inhibitors and stabilize the thrombin so it can be used hours after production.²¹ A concern that has been raised by this method, however, is that residual ethanol content may have an impact on platelet activation, cell proliferation, and the viability of tissue cells close to the applied platelet gel. The aim of this study was to compare the influence of thrombin prepared by the TPD or BT on the release of growth factors from the platelet gel and the effect of the platelet gel on in vitro cell proliferation and viability. An overview of the study design can be seen in Figure 1. The results suggest that residual ethanol in the TPD-produced thrombin does not affect any of the tested parameters and has similar characteristics as commercially available BT.

Materials and Methods

PREPARATION OF PLATELET-RICH PLASMA

Citrated whole blood was obtained from healthy volunteers according to established procedures for the collection of human blood products. The blood was centrifuged at 1,500g for 10 minutes to separate the PRP from the red cells. The PRP was harvested

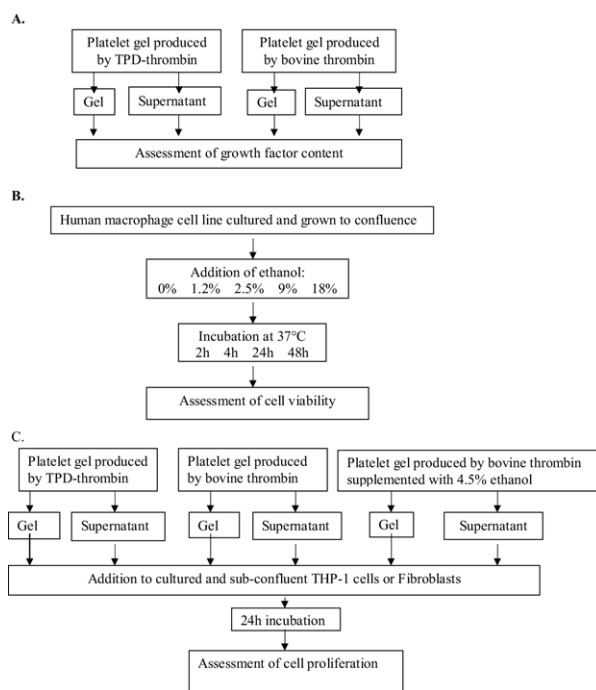


FIGURE 1. An overview of the study design. A, Study design of the assessment of growth factor content in the platelet gel and the platelet gel supernatant. B, Design of the study assessing cell viability after addition of ethanol at different concentrations. C, Design of the study assessing cell proliferation after stimulation with either platelet gel or platelet gel supernatant. THP-1, human macrophage cell line from the American Type Tissue Culture Collection (ATCC, Manassas, VA); TPD, Thrombin Processing Device (Thermogenesis Corp, Rancho Cordova, CA).

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together with the white cells and the upper part of the red cell layer, and used within 1 hour of separation.

PREPARATION OF TPD THROMBIN

For the production of thrombin, the TPD was used. It consists of 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 (final concentration 7.2% and 19% mmol/L, respectively). Fresh frozen plasma was obtained from San Bernadino Blood Bank (San Bernadino, CA) and South Florida Blood Bank (St Petersburg, FL). The plasma was donated by healthy volunteers and processed according to local established procedures for the collection and processing of human blood products. Each unit was thawed in a plasma thawer (MT-202; Thermogenesis Corp, Rancho Cordova, CA) within 1 hour of initiation of thrombin production.

Eleven mL of fresh frozen plasma and 4 mL thrombin reagent was added to the TPD reaction chamber. After mixing and incubating for 20 minutes at room

temperature, the TPD was agitated to break any formed fibrinogen clots and incubated for an additional 5 minutes. The TPD-produced thrombin was harvested, in 3 mL aliquot samples and frozen at -80°C until use. Bovine thrombin (Thrombin-JMI; GenTrac Inc, Middleton, WI), commonly used in surgery, was used as a control. This thrombin was commercially produced by the Cohn fractionation method.

THROMBIN ACTIVITY, QUANTITATIVE ASSESSMENT OF THE THROMBIN

Thrombin activity was analyzed using the modified Clauss method.²² Briefly, 200 μL of a 2 mg/mL prewarmed (37°C) solution of fibrinogen (Sigma, St Louis, MO) solution was added to 100 μL of thrombin sample. The time required for clot formation was recorded using a fibrometer (FibroSystem; Becton-Dickinson, Franklin Lakes, NJ). The thrombin activity in the samples was determined by correlating the time to clot formation to a standard curve generated with titrations of thrombin (Biopool US Inc, Ventura, CA). As each time point on the standard curve corresponds to the thrombin activity needed to clot the standard concentration of fibrinogen, the thrombin activity in an unknown sample can be extrapolated from the time to clot formation.

PREPARATION OF PLATELET GELS

Two different ratios of PRP to thrombin were used to produce platelet gels; 3.3 to 1 and 7 to 1. Platelet gels were prepared by adding TPD-thrombin and BT to PRP at the indicated ratios. The platelet gels prepared with TPD-thrombin had a final ethanol concentration of 4.2% and 2.25% (ratios 3.3:1 and 7:1, respectively). As a control for an ethanol effect, platelets gels were also prepared using BT with added ethanol (final concentration 4.5%). When clotting was complete, the clot and the supernatant were separated by centrifugation (1,000g for 5 min at 4°C). The supernatants were harvested and frozen at -80°C until use. The clots were homogenized (PRO 200 homogenizer; PRO Scientific Inc, Oxford, CT) using a medium speed for 5 minutes at 4°C and the homogenates were frozen at -80°C until use. Both supernatants and homogenates were used in the cytokine assays and supernatants in the cell proliferation and viability assays.

ASSESSMENT OF GROWTH FACTOR CONTENT IN PLATELET GELS AND PLATELET GEL SUPERNATANT

The presence of PDGF-B and TGF- β was tested in both the supernatant and the homogenized clots from the platelet gels using commercially available ELISA kits (R&D Systems, Minneapolis, MN). The positive control consisted of a totally homogenized platelet gel

(both supernatant and clot) and represented the total available cytokines in the preparation. A negative control was prepared by first inactivating the platelets in the PRP by adding Prostaglandin- E_1 (Sigma). The presence of cytokines within the Prostaglandin- E_1 -treated supernatants of the gel was a measure of inhibition of cytokine release. See Figure 1A for an overview of the study design.

ASSESSMENT OF CELL VIABILITY AND PROLIFERATION

Cell Lines

The following cell types were used for assaying influence on proliferation and viability: Fibroblasts (WS1) and the human macrophage line (THP-1) were obtained from the American Type Tissue Culture Collection (ATCC, Manassas, Virginia). The cell lines were maintained with RPMI-1640 containing 10% fetal calf serum (FCS; Gibco BRL, Grand Island, NY), 100 $\mu\text{g}/\text{mL}$ penicillin/streptomycin/fungizone, 100 mmol/L L-glutamine (Gibco BRL), and 5×10^{-5} mol/L 2-mercaptoethanol (cRPMI; Sigma Aldrich, St Louis, MO).

Cell Culture

Cells were grown to confluence and then removed by washing (THP-1) or trypsinization and washing (fibroblasts), and then 100 μL of 10^5 cells were seeded in triplicate into 96-well flat-bottom plates with the indicated concentrations of ethanol in platelet gel or supernatant to a final volume of 150 μL and incubated for the indicated times at 37°C in a humidified CO_2 (5% final) incubator (Precision Instrument; Fisher Scientific, Markham, Ontario, Canada). The cells were then tested for viability or their ability to proliferate.

Cell Viability

Cell viability in all experiments was assessed by adding propidium iodide to the wells for 10 minutes and acquiring them on a FACSsort flow cytometer (Becton-Dickinson, San Jose, CA) equipped with an Argon laser operating at 15 mW; 10,000 events were acquired using an electronic cellular (lymphocyte) gate based on forward and side scatter, and FL2 fluorescence was analyzed using Cellquest software (Becton-Dickinson).²³ See Figure 1B for an overview of the study design.

Cell Proliferation

Cell proliferation was measured by ^3H -thymidine incorporation. Briefly, at 0 hours and 24 hours incubation, ^3H -thymidine (1 μCi) was added to the indicated cell culture wells and incubated for an additional 24 hours. The cells were then harvested onto filter paper and incorporation of ^3H -thymidine was measured using a liquid scintillation counter (1209

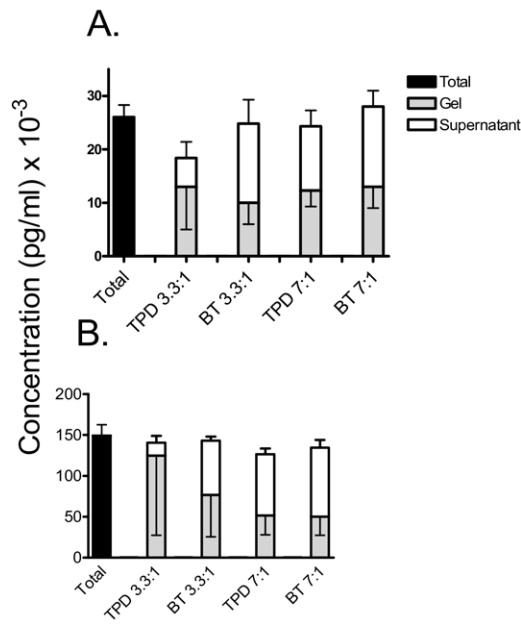


FIGURE 2. The proportion of growth factors (A, platelet-derived growth factor beta polypeptide; B, transforming growth factor-β) derived from platelets found in the total homogenate (black bars), in the gel (gray bars) and the supernatant (white bars) after preparation of platelet gels using TPD (Thrombin Processing Device [Thermogenesis Corp, Rancho Cordova, CA]) thrombin or bovine thrombin (BT) in 3.3 to 1 and 7 to 1 platelet-rich plasma to thrombin ratios.

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Rack beta; LKB/Wallac Oy, Turku, Finland). See Figure 1C for an overview of the study design.

STATISTICAL ANALYSIS

All results are presented as mean and standard deviation. Significance between groups was assessed using the Student *t* test or Wilcoxon signed rank test, where appropriate.

Results

The PRP used for platelet gel production had a platelet concentration of greater than 600 × 10³ per μL and a white cell concentration of greater than 15 × 10³ per μL. The TPD-thrombin had a thrombin activity of 81 ± 20 IU per mL. The BT used in the experiments had a concentration of 50 IU per mL.

ASSESSMENT OF GROWTH FACTOR CONTENT IN PLATELET GELS AND PLATELET GEL SUPERNATANT

In the platelet gel homogenates produced using TPD-thrombin with a 3.3 to 1 ratio of PRP to thrombin, there was a lower amount of PDGF-B and TGF-β in the supernatants compared with what was found in the supernatant of the platelet gel produced using BT (Fig 2). However, the amount of released PDGF-B and TGF-β in the supernatants using a 7 to 1 PRP to

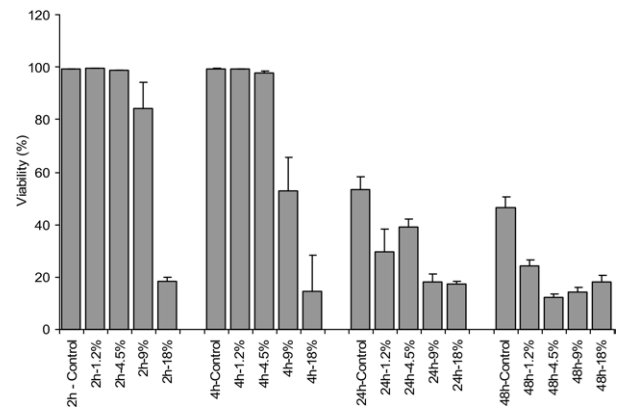


FIGURE 3. The effect of ethanol on the viability of THP-1 (human macrophage cell line from the American Type Tissue Culture Collection [ATCC, Manassas, VA]) cells. The cells were exposed to different concentrations of ethanol for 2, 4, 24, or 48 hours. The control cells were not exposed to ethanol. The viability of the control cells after 2 hours was defined as 100%.

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thrombin dilution was similar for both TPD-thrombin and BT (Fig 2).

CELL VIABILITY

To examine the threshold concentration of ethanol required to inhibit viability of THP-1 cells or fibroblasts, titrations of ethanol were added to cultures and viability was measured at various time points. Exposing the cells to 4.2% ethanol (final) for 4 or 24 hours had little effect on the viability (Fig 3). However, significant death occurred in the cultures of

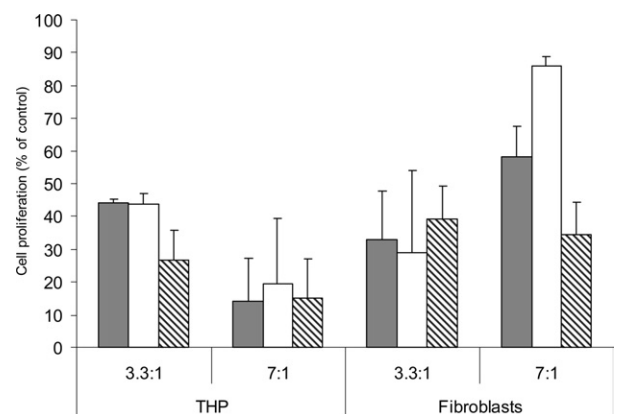


FIGURE 4. Proliferation of THP-1 (human macrophage cell line from the American Type Tissue Culture Collection [ATCC, Manassas, Virginia]) cells and fibroblasts after 48 hours exposure to supernatants from platelet gels prepared using TPD (Thrombin Processing Device [Thermogenesis Corp, Rancho Cordova, CA]) thrombin (gray bars), bovine thrombin (white bars) and bovine thrombin supplemented with ethanol (hatched bars).

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both cell types when exposed to 9% ethanol for 4 hours (Fig 3).

CELL PROLIFERATION

The proliferation of THP-1 cells and fibroblasts after exposure to the different platelet gels or their supernatants was assessed using ^3H -thymidine incorporation. There was no difference between the proliferation of THP-1 cells and background using supernatants from platelet gels produced using TPD-thrombin, BT, or BT with 4.5% ethanol added, at PRP to thrombin ratios of 3.3 to 1 or 7 to 1 after 48-hour culture (Fig 4). Fibroblasts, however, proliferated significantly using both ratios and there was no difference in proliferation between the wells incubated with supernatants produced using either TPD-thrombin, BT, or BT with 4.2% ethanol added (Fig 4).

Discussion

Platelet gels are commonly used in surgery to enhance wound healing and osteogenesis. The gels are produced by combining PRP with thrombin, typically from a bovine source. By introducing the TPD, it has become possible to produce stable thrombin from the patient's own blood, thus avoiding bovine exposure and possible adverse reactions. In this study we investigated the effect of residual ethanol in TPD-thrombin on release of growth factors from platelet gels and on the ability of the platelet gels or their supernatants to affect in vitro cell viability and proliferation.

When platelet gels were prepared using a ratio of PRP to thrombin of 7 to 1 with both TPD-thrombin and BT approximately 50% of the total available growth factors PDGF-B and TGF- β were found in the supernatant. However, using a ratio of 3.3 to 1, the supernatant of the platelet gel produced with TPD-thrombin had a lower PDGF-B and TGF- β content compared with gel produced with BT (PDGF-B, 30% and 65%, respectively; and TGF- β , 15% and 40%, respectively). The final ethanol concentration in a platelet gel produced with TPD-thrombin at a 3.3 to 1 ratio is approximately 4%. As ethanol is known to inactivate platelets,^{24,25} it remains to be investigated if this is the reason for the lower release compared with BT containing no ethanol. The interpretation of these results may have to be taken with some caution as there is a large variation in the results, especially in the samples taken from the gel. This may be due to the possible inactivation of platelets by ethanol making the method chosen for recovery of growth factors from the gel (homogenization and freezing) less adequate. On the other hand, if there is less release of growth factors, it

may be potentially beneficial for the patient. As growth factors already released from the platelets will quickly break down and diffuse into the tissue at the wound site, having more growth factors available in the gel, for slower release over time, may be an advantage. In addition, it has been shown that platelet aggregation can be inhibited by PDGF, thus a high concentration of PDGF at the wound site may have a negative feedback effect on the activation of circulating platelets.^{26,27} However, this still remains to be determined.

The effect of ethanol on cell viability was tested using THP-1 cells, a monocytic cell line that is extremely phagocytic. This cell line was chosen because monocytes are one of the first cell types that migrate to and proliferate at the wound site; they release cytokines that attract cells pivotal for wound healing, eg, platelets, granulocytes, fibroblasts, and epithelial cells. Sustaining an ethanol concentration of 4.5% for 4 hours had no effect on the cell viability and exposing the cells for 24 hours only decreased the viability with approximately 25% compared with the control. Several studies have reported various effects of using different ethanol concentrations and exposure times on different types of cell lines.²⁸⁻³³ The results vary, but overall the negative effect increased with increasing concentrations of ethanol and time of exposure. For example, Stephens et al²⁹ showed that proliferation of cultured fibroblasts was significantly affected when an ethanol concentration of greater than 5% for 48 hours was used. Using a pig model, De Somer et al³³ investigated the effect of 1.18% and 3.8% ethanol on cell damage when applied on the ischiadic nerve. They did not find any histological or structural changes in either group and it thus appears that ethanol concentrations at this low range do not cause impairment at the application site. The in vitro tissue culture evaluations are the "worst case" conditions of ethanol exposure and provide high assurance that residual ethanol will not be the source of significant cytotoxicity for the in vivo applications due to physiologic clearance mechanism.

The proliferation of fibroblasts was similar using supernatants from platelet gels prepared by TPD-thrombin and BT at both 3.3 to 1 and 7 to 1 PRP to thrombin ratios (Fig 3). However, this cell type proliferated marginally better using supernatants from PRP-thrombin ratio 7 to 1 compared with 3.3 to 1. This could be due to the somewhat higher concentration of PDGF-B in the supernatant in the 7 to 1 supernatant as PDGF-B is a very potent stimulator of growth for fibroblasts.³⁴ On the other hand, using THP-1 cells, we did not detect any proliferation above the baseline of unstimulated

cells. The monocytic THP-1 cell is less susceptible to stimulation of PDGF-B and more responsive to TGF- β , compared with fibroblasts. At high concentrations, as in this case, TGF- β has been shown to be an inhibitor of proliferation for hematopoietic cells, such as THP-1 cells.³⁵ This could explain the results as the concentration of TGF- β was higher in the supernatants from the PRP-thrombin ratio 3.3 to 1 compared with the ratio 7 to 1.

To investigate the ethanol effect, we added ethanol to BT and produced platelet gels as previously described. Adding ethanol did not significantly change the proliferation of THP-1 cells or fibroblasts compared with using TPD-thrombin or BT at a PRP to thrombin ratio of 3.3 to 1. At the ratio of 7 to 1, the proliferation was decreased compared with using both TPD-thrombin and BT. Comparing the 3.3 to 1 and 7 to 1 PRP to thrombin ratios, the proliferation was similar. As we were using the supernatants, this could indicate that more growth factors were retained in the gel or in the platelets, as the platelets could have been inhibited in their release by the ethanol. However, this remains to be investigated.

In summary, these data demonstrate that cultured cells related to wound healing and exposed to the supernatants from platelet gels prepared by either TPD-thrombin or BT have similar viabilities and fibroblasts, at least, proliferated in a similar manner. It thus appears that platelet gels produced with TPD-thrombin have similar characteristics as commercially available BT.³⁶ The potential benefit of slow release of growth factors from the platelet gel to the wound site still has to be investigated.

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