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ACTIVATION OF PLATELET-RICH PLASMA USING SOLUBLE TYPE I COLLAGEN

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Abstract

PURPOSE—Platelet-rich plasma (PRP) has recently been found to be a useful delivery system for growth factors important in oral tissue healing. However, application of PRP in a liquid form to a wound site within the oral cavity can be complicated by significant loss of the PRP into the surrounding oral space unless gelation via the clotting mechanism is accomplished. Gelation is currently accomplished using bovine thrombin; however, rare but serious complications of this method have led to the search for alternative clotting mechanisms, including the use of soluble collagen as a clotting activator. In this paper, our hypothesis was that soluble Type I collagen would be as effective as bovine thrombin in causing clotting of the PRP and of stimulating growth factor release from the platelets and granulocytes.

MATERIALS AND METHODS—PRP from human donors was clotted using Type I collagen or bovine thrombin. Clot retraction was determined by measuring clot diameters over time. The release of PDGF-AB, TGF- β 1 and VEGF from both types of clots was measured over 10 days using ELISA.

RESULTS—Clots formed using Type I collagen had far less retraction than those formed with bovine thrombin. Bovine thrombin and Type I collagen stimulated similar release of PDGF-AB and VEGF between 1 and 10 days; however, thrombin activation resulted in a greater release of TGF- β 1 during the first five days after activation.

CONCLUSIONS—The use of Type I collagen to activate clotting of PRP may be a safe and effective alternative to bovine thrombin. The use of collagen results in less clot retraction and equal release of PDGF-AB and VEGF when compared to currently available methods of clot activation.

Keywords

Platelet-rich Plasma; collagen; thrombin; PDGF-AB; TGF- β 1; VEGF

INTRODUCTION

Platelet-rich plasma has recently been found to be a useful delivery system for growth factors important in oral and maxillofacial surgery^{1–4}. When applied to a clinical defect, such as the wound site of a ligament, it can stimulate healing^{5,6}. PRP can be used to increase local

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concentrations of active PDGF-AB⁷ and TGF- β 1⁷⁻⁹ by over 300%^{7,9,10}. As seen in vitro, cytokines released by platelet concentrates can result in increased fibroblast DNA synthesis^{7,11}, up-regulation of collagen production¹² and changes in collagen organization¹³.

However, application of PRP in a liquid form to a wound site within the oral cavity or other relatively uncontained wound site can be complicated by significant loss of the PRP into the surrounding space unless gelation via the clotting mechanism is accomplished. Gelation is currently accomplished using bovine thrombin. However, alternatives to bovine thrombin have been sought due to the clinical complications of the development of antibodies. While bovine thrombin is a potent platelet activator¹⁶, it also causes the development of antibodies against thrombin, prothrombin, factor V, and cardiolipin with resultant clinical problems that range from severe postoperative bleeding to an autoimmune syndrome similar to lupus in animal studies¹⁷. These problems are rarely seen, but can be devastating when they occur. The use of bovine thrombin also results in impaired migration of fibroblasts through collagen-PRP clots, as well as impaired strength of the clots,¹⁸ so alternatives to thrombin have been of great clinical interest^{19,20}. In addition, the high degree of retraction seen with thrombin-activated clots makes them difficult for use in wound-space-filling applications.

The objective of this study was to investigate the use of Type I collagen as an alternative to bovine thrombin as a PRP clot activator. Our hypothesis was that Type I collagen and bovine thrombin would be equally effective in causing clotting of the PRP and of stimulating growth factor release from the platelets and granulocytes, and that the use of collagen would stabilize the PRP clots against excessive retraction. To test this hypothesis, we obtained samples of PRP from human donors, induced platelet activation and gelation using either bovine thrombin or Type I collagen, and measured the release of platelet-associated growth factors PDGF-AB and TGF- β 1, as well as the release of granulocyte-mediated growth factor VEGF using enzyme-linked immunosorbent assays (ELISA). Clot retraction was measured as well for both the thrombin- and collagen-activated gels.

MATERIALS AND METHODS

Preparation of Platelet-rich Plasma

IRB approvals were obtained prior to beginning the study. Three hundred milliliters of whole blood was drawn from each of four hematologically normal volunteers meeting all criterion of the American Association of Blood Banks (Food and Drug Administration, Center for Biologics Evaluation and Research). The blood from each volunteer was drawn into five 60 cc syringes each containing 6 cc of acid-citrate dextrose. The five syringes for each volunteer were pooled in a 300 milliliter transfer pack at the Center for Blood Research (Boston, MA). PRP was produced using the Harvest Smart PreP2 SystemTM (Harvest Technologies, Plymouth, MA) by the method recommended by the manufacturer. Sixty ml of anticoagulated blood was transferred to the blood chamber of the device, and 2ml ACD was placed in the plasma chamber of the disposable blood processor (DP). Following the separation of plasma from the red blood cells by centrifugation, the platelets, plasma and white blood cells were decanted into the plasma chamber. A second centrifugation step was used to form a pellet of platelet concentrate in the bottom of the plasma chamber. Approximately 2/3 of the platelet poor plasma (PPP) was removed and the platelet concentrate (PC) was then resuspended in 10 ml of PPP.

Platelet Activation: Type I Collagen

Rat tails were obtained from control breeder rats undergoing euthanasia for other Institutional Animal Care and Use Committee approved studies. The rat-tail tendons were sterilely harvested, minced, and solubilized in an acidified enzyme solution to obtain the acid soluble

collagen. This solution was neutralized using a solution containing HEPES Buffer (Cellgro, Mediatech, Inc., Herndon, VA), Ham's F-10 medium (MP Biomedicals, LCC, Aurora, OH), Antibiotic-Antimycotic solution (Cellgro, Mediatech, Inc.), sterile water, and 7.5% sodium bicarbonate (Cambrex BioScience Walkersville, Inc., Walkersville, MD). To create the collagen-PRP gel, an equal volume of PRP and collagen were combined.

Platelet Activation: Exogenous Thrombin Group

Five milliliters of calcium chloride (100 mg/ml) were added to 5,000 IU bovine thrombin (Bovine Thrombin – JMI, Jones Pharma Inc, Bristol, VA) to produce a 1,000 IU/ml solution. 80 microliters of the thrombin solution was then added to 720 microliters of the Platelet Concentrate group for each sample to produce 0.8 cc of a 1:10 dilution.

Culture conditions—For the collagen and thrombin clots, duplicate samples for each donor (n=4) were injected into 2ml centrifuge tubes and allowed to form a clot. Clots were weighed and placed in a 37° C incubator for 20 minutes prior to transfer to sterile 12-well plates. One milliliter of Dulbecco's Modified Eagle's Medium (DMEM, Cat# 10013CV, Cellgro, Mediatech, Inc., Herndon, VA) with 2% Antibiotic-Antimycotic solution (Cellgro, Mediatech, Inc., Herndon, VA) was added to each clot. Samples were cultured in a 37° C humidified incubator.

Clot retraction measurements—Four clots of bovine thrombin activated PRP and four clots of collagen activated PRP were made and clot length and width measured as a function of time in culture between 0 and 10 days.

Measurement of Growth Factor Levels—Interval release of TGF- β 1, PDGF-AB, and VEGF from each gel was measured at 12 hours and 1, 3, 5, 7 and 10 days. At each time point, one ml of media was aspirated from around each sample and replaced with one ml of fresh media. Media samples were stored in 1.3 ml cryovials in a -80°C freezer until all samples were collected. Concentrations of human PDGF-AB, TGF- β 1 and VEGF were determined using the commercially available Quantikine colorimetric sandwich ELISA kits (R&D Systems, Minneapolis, MN). Assays were performed in duplicate on media samples as described in the instructions of the manufacturer. Dilutions of 1:20 (12 hour samples) and 1:10 (day 1, day 3, day 5, day 7 and day 10 samples) were used for samples in the PDGF-AB assay; a dilution of 1:10 was used for all samples in the TGF β 1 assay; and no dilution was used for the VEGF assay. These dilutions were accounted for in analysis. TGF β 1 was assayed after acid activation of the plasma by adding 20 microliters of 1N HCl to 40 microliters of media sample. The reaction solution was mixed and incubated at room temperature for 10 minutes before it was neutralized by with microliters of 1.2N of NaOH/0.5 M HEPES and diluted to 1:10 in calibrator diluent before it was added to the ELISA plate.

For each growth factor, the standard curve was produced with concentrations of 0, 31.2, 62.5, 125, 250, 500, 1000 and 2000 pg/ml. The color change of the final reaction was measured at a wavelength of 450 nm for the optical density and the concentration vs. absorbance was linear using a four parameter logistic fit curve. The reported minimal detection limit of TGF- β 1 was 4.6 pg/ml, 9.0 pg/ml for VEGF and 1.7 pg/ml for PDGF-AB. After readings were obtained, the total reading for each sample was divided by the weight of PRP, and all results reported are per gram of PRP.

Due to the media sampling technique described above, growth factor concentrations reported in the results section reflect the growth factor release in the time period since the prior media change. For 12 hours and day 1, this represents 12-hour release and for day 3, day 5 and day 7, it represents 48 hour release.

Statistical Analysis

Repeated-measures ANOVA was used to compare the clot retraction and growth factor release of the collagen-PRP clots with those of the bovine thrombin-PRP clots, with values of $p < 0.05$ considered statistically significant.

RESULTS

CLOT RETRACTION

The clots formed using bovine thrombin had significantly greater retraction within the first twenty four hours than the clots activated with Type I collagen (Figure 1; $133 \pm 1.7 \text{ mm}^2$ vs. $74 \pm 23 \text{ mm}^2$; mean \pm standard deviation, repeated measures ANOVA, $p < 0.003$). The clots in the thrombin group lost 93% of their volume, while the clots in the collagen group retained 50% of their original volume. This difference persisted out to five days. After five days in culture, the thrombin-activated clots had disintegrated to the point their dimensions could no longer be measured, while the collagen-activated clots retained their integrity and had a negligible change in size between day 5 and day 10.

GROWTH FACTOR RELEASE FROM PRP PREPARED WITH THROMBIN OR TYPE I COLLAGEN

The thrombin gels had significantly higher PDGF-AB release than the collagen gels (repeated measures ANOVA, $p < 0.0005$), a difference which was most prominent at the early time points where the release from the thrombin gels was more than three times as high as the collagen group at twelve hours ($628 \pm 121 \text{ ng/g PRP}$ vs. $160 \pm 14 \text{ ng/g PRP}$; mean \pm SD) and almost twice as high at 24 hours ($78 \pm 63 \text{ ng/g PRP}$ vs. $44 \pm 14 \text{ ng/g PRP}$; mean \pm SD). There was also a statistically significant difference in total release of PDGF-AB ($752 \pm 140 \text{ ng/g PRP}$ vs. $231 \pm 16 \text{ ng/g PRP}$; mean \pm SD, $p < 0.0005$). When only time points after 24 hours were included in the analysis, there was no significant difference between the thrombin and collagen groups (Figure 2; repeated measures ANOVA, $p > 0.15$).

The thrombin gels had significantly higher release of TGF- β 1 than the collagen gels (Figure 3; repeated measures ANOVA, $p < 0.03$). This difference was most notable in the first five days of culture, particularly at twelve hours ($456 \pm 144 \text{ ng/g PRP}$ vs. $293 \pm 90 \text{ ng/g PRP}$; mean \pm SD) and at 24 hours ($73 \pm 51 \text{ ng/g PRP}$ vs. $93 \pm 44 \text{ ng/g PRP}$; mean \pm SD). The thrombin gels also had higher cumulative release of TGF- β 1 than the collagen gels over the entire 10 day period of culture ($1219 \pm 353 \text{ ng/g PRP}$ vs. $603 \pm 114 \text{ ng/g PRP}$; mean \pm SD, $p < 0.03$).

VEGF release was similar in the thrombin and collagen groups (Figure 4; repeated measures ANOVA, $p > 0.17$), with persistent release over the 10 day time frame, with $295 \pm 160 \text{ pg/g PRP}$ (mean \pm SEM) continuing to be eluted from the thrombin group and $184 \pm 110 \text{ pg/g PRP}$ (mean \pm SEM) still eluting from the collagen group at the 10 day time point. There was no significant difference in total release of VEGF between the thrombin and collagen gels over the ten day time period ($6.7 \pm 4.2 \text{ ng/g PRP}$ vs. $3.3 \pm 1.4 \text{ ng/g PRP}$; mean \pm SD, $p > 0.15$).

DISCUSSION

This study investigated the use of Type I collagen as an alternative to bovine thrombin for PRP activation. The results reported here show that Type I collagen is as effective as thrombin in stimulating release of PDGF-AB and VEGF at time points between 1 and 10 days, and stabilizes the PRP clot against excessive retraction. These findings suggest that Type I collagen may

provide a safe, readily available and inexpensive alternative to bovine thrombin in the clinical applications of PRP clots.

Growth factors such as TGF- β 1 and PDGF-AB are thought to play an important role in the regulation of extracellular matrix deposition and fibrosis in a wide range of tissues, including ligaments²². PDGF-AB has a normal plasma level of approximately 41 ng/ml⁷. In this paper, the PDGF-AB release from the collagen-PRP gels within the first 12 hours averaged 160 ng released per ml of PRP. At 3 days, both groups continued to elute PDGF from the gels at far lower levels: between 1 and 14 ng/ml of PRP over 48 hours for the collagen gels and between 2 and 27 ng/ml of PRP over 48 hours for the thrombin gels. Thus, the initial release of PDGF-AB into the wound milieu is at supra-physiologic levels, but quickly drops off to levels well below that in the circulating plasma for both groups. Whether the supra-physiologic PDGF-AB levels released by these gels is helpful or harmful to the wound healing process remains to be determined. The usefulness of PDGF in stimulating wound healing has been shown for epidermal wounds, where a dose-dependent response was seen, with higher levels of partially purified PDGF (ppPDGF) was found to stimulate the formation of new connective tissue when the ppPDGF was applied in ranges from 1 to 4 ng per square millimeter of wound²³.

TGF- β 1 is found in human plasma in both the latent and activated forms, with mean latent levels of approximately 35 to 40 ng/ml^{7,9} and activated forms reported to range from 1.23 to 5.3 ng/ml²⁴⁻²⁶. Increases of 2-3 times the normal plasma value of active TGF- β 1 has been shown to cause renal fibrosis²⁷ and increased cell influx and matrix deposition into subcutaneous sponges²⁸ in mice, and in humans, elevated levels are associated with coronary artery disease²⁴. Thus, changes in activated TGF- β 1 concentration as small as 1 ng/ml can have clinically important biologic effects on fibrosis of multiple tissues. In this paper, we report that both collagen and thrombin can stimulate continuing platelet release of TGF- β 1 in the range of 23 to 35 ng/ml PRP over 48 hours at ten days after platelet activation. This suggests that the sustained release of TGF- β 1 from both thrombin-PRP and collagen-PRP gels is in a clinically significant range.

Whether the increased TGF- β 1 release seen from the thrombin clots is beneficial or detrimental also requires further study. TGF- β 1 release is associated with fibrosis, a process which may be helpful to ligament or articular cartilage healing, but harmful to the overall joint if it results in synovial tissue thickening or arthrofibrosis²⁹. Therefore, the optimum range of TGF- β 1 in vivo is likely to be within a relatively narrow range that remains to be determined.

The major contribution of VEGF to the wound healing process is currently thought to be in stimulating angiogenesis; however, the roles of this complex molecule in wound healing are still being defined³⁰ with roles postulated in extracellular matrix production³¹, cell recruitment³² and cellular proliferation³³. VEGF is found in concentrations of 97 to 266 pg/ml in normal control serum^{34,35}, and is elevated in the serum of patients with rheumatoid arthritis (590 pg/ml), and osteoarthritis (287 pg/ml)³⁴. It is also found to be elevated in the synovial fluid of patients with RA (1216 \pm 211 pg/ml), and osteoarthritis (434 \pm 131 pg/ml), where normal synovial fluid levels are 335 \pm 146 pg/ml³⁶. Exudates from acute wounds contain between 1 and 2 ng/ml of VEGF while exudates from inflamed wounds contain between 4 and 8 ng/ml of VEGF up to 8 days after wounding³⁷. Thus, the levels of initial release of VEGF from collagen clots of 646 pg/ml of PRP and from thrombin clots of 1207 pg/ml of PRP is in a range approaching that of differences between normal and diseased serum, synovial fluid, and wound exudates and may be clinically relevant.

Collagen as a platelet activator also shows promise when compared with other thrombin alternatives, such as thrombin receptor agonist peptide (TRAP)²⁰. In a recent report, TRAP has been shown to stimulate the release of approximately 40 ng/mg PRP of PDGF-AB in the

first 24 hours after clot formation²⁰, while the collagen-activated clots reported here resulted in release of 200 ng/mg PRP of PDGF-AB in the same time frame. This increased release was seen to extend to 7 days for PDGF-AB, and was also seen with TGF- β 1, with collagen-activated clots releasing over 390 ng/mg PRP over the first 24 hours, while TRAP released 14 ng/mg PRP²⁰, and collagen continuing to stimulate greater release at 3 to 7 days after clot formation (70 ng/mg PRP vs. 2 ng/mg PRP over 4 days). Although the increased release seen with collagen over TRAP could be construed as premature loss of these growth factors from the wound site, recent work has shown that collagen-PRP clots are able to retain TGF- β 1 and PDGF-A in wounds in vivo for as long as six weeks after clot placement in the wound³⁸, and to stimulate healing both histologically and biomechanically^{5,21}. The results here at 24 hours are also comparable to the results published previously for “ITA gelling agent” at 24 hours, where use of that agent resulted in release of PDGF-AB and TGF-b1 from the PRP clots at levels similar to thrombin-PRP clots {Landesberg, 2000 #2071}.

The collagen-activated clots retracted approximately 50% in the first 24 hours, and then stabilized in size over the next 10 days. This is in comparison to the thrombin-activated clots which retracted over 90% in the first 24 hours, then disintegrated after 5 days in culture with serum. Thus, the clots formed using collagen have far less retraction than those created with bovine thrombin, a characteristic which may be desirable as PRP clots are increasingly used for tissue engineering applications where premature degradation and excessive clot retraction may inhibit wound filling and healing. However, the retraction seen with collagen was greater than that reported for TRAP activated clots, where only a 15% loss in diameter was measured in the first 24 hours²⁰.

In conclusion, both thrombin and Type I collagen are potent stimulators of platelet and granulocyte activation and are able to stimulate high initial release as well as sustained release over 10 days from a PRP clot. Collagen has the additional advantage of resulting in less retraction of the PRP clots over time than thrombin, a feature which may be important in the use of PRP clots as wound healing scaffolds. Although the levels of release measured in this study are within the range of physiologic levels, future work is needed to determine if these amounts will have a significant effect on in vivo wound healing.

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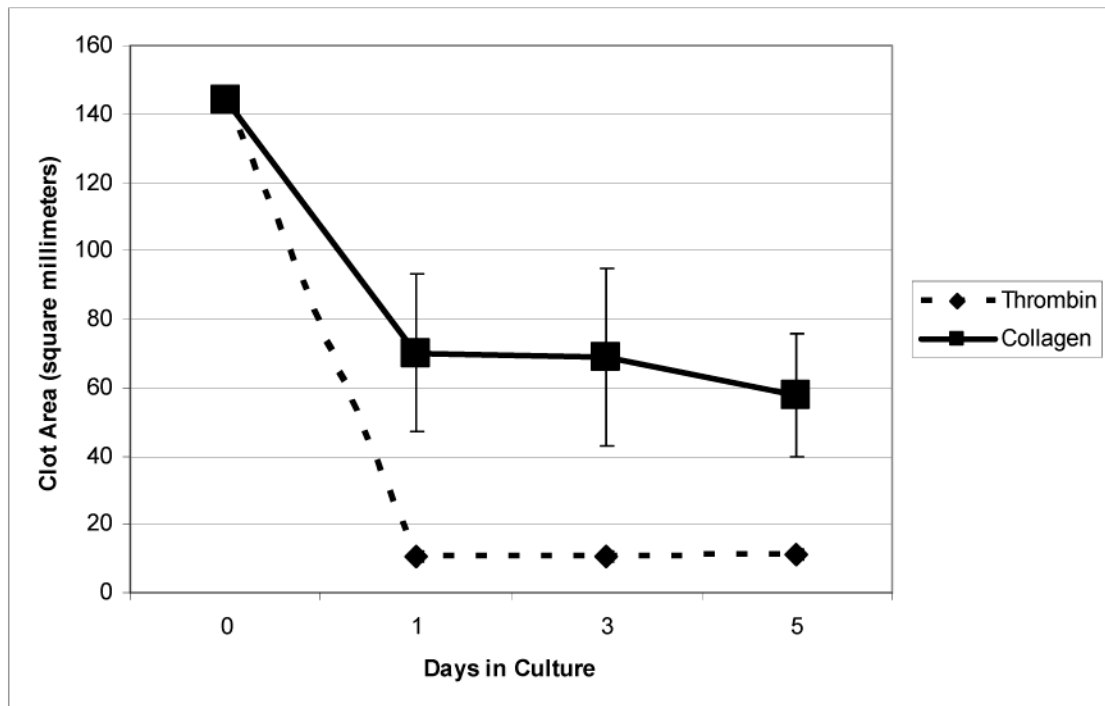


Figure 1.

Clot Retraction of bovine thrombin activated clots and Type I collagen activated clots. The thrombin-PRP clots had significantly greater retraction within the first twenty four hours than the clots activated with Type I collagen ($133 \pm 1.7 \text{ mm}^2$ vs $74 \pm 23 \text{ mm}^2$; mean \pm st dev, repeated measures ANOVA, $p < 0.003$). Points represent the average value at each time point, error bars represent the standard deviation for each group ($n=4$) at each time point.

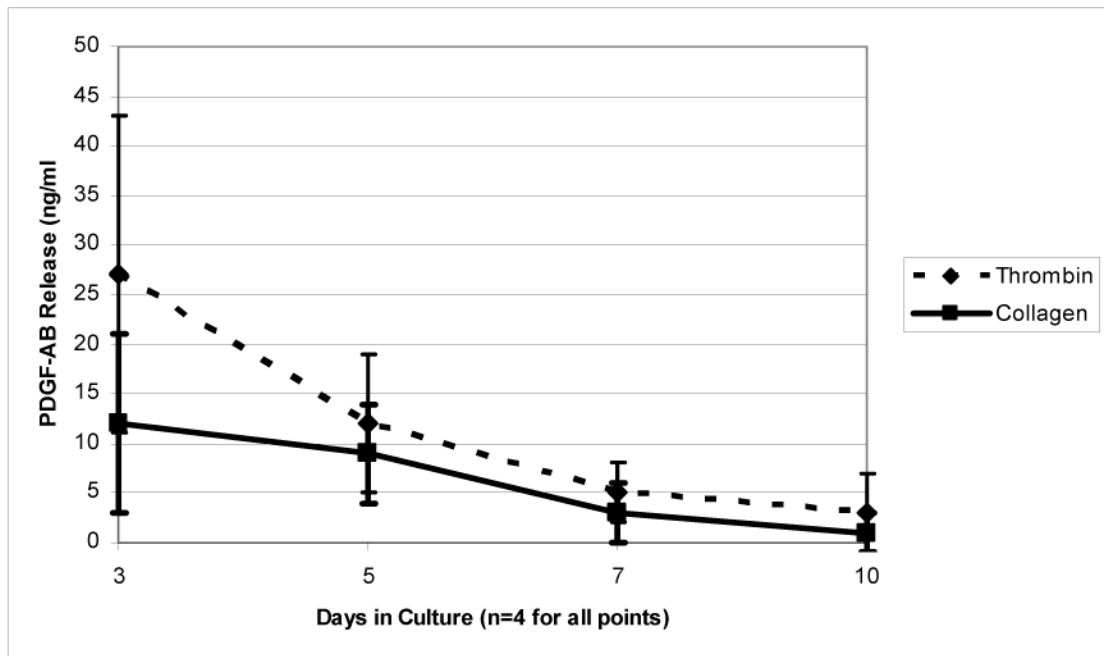


Figure 2. PDGF-AB release from thrombin and collagen PRP clots at time points greater than 24 hours. No significant difference was observed between groups at these later time points ($p > 0.15$). Points represent the average value at each time point, error bars represent the standard deviation for each group ($n=4$) at each time point.

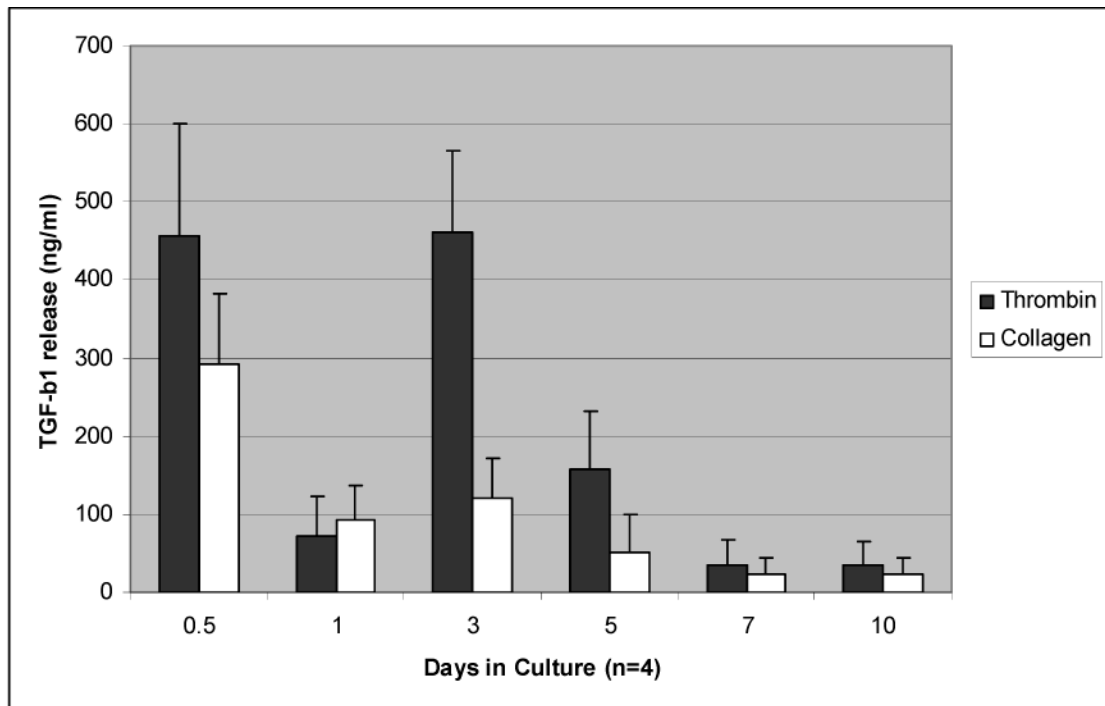


Figure 3. TGF- β 1 release from thrombin and collagen PRP clots at time points between 12 hours and 10 days. A significant difference was observed between groups at all time points (repeated measures ANOVA, $p < 0.03$). Bars represent the average value at each time point, error bars represent the standard deviation for each group ($n=4$) at each time point.

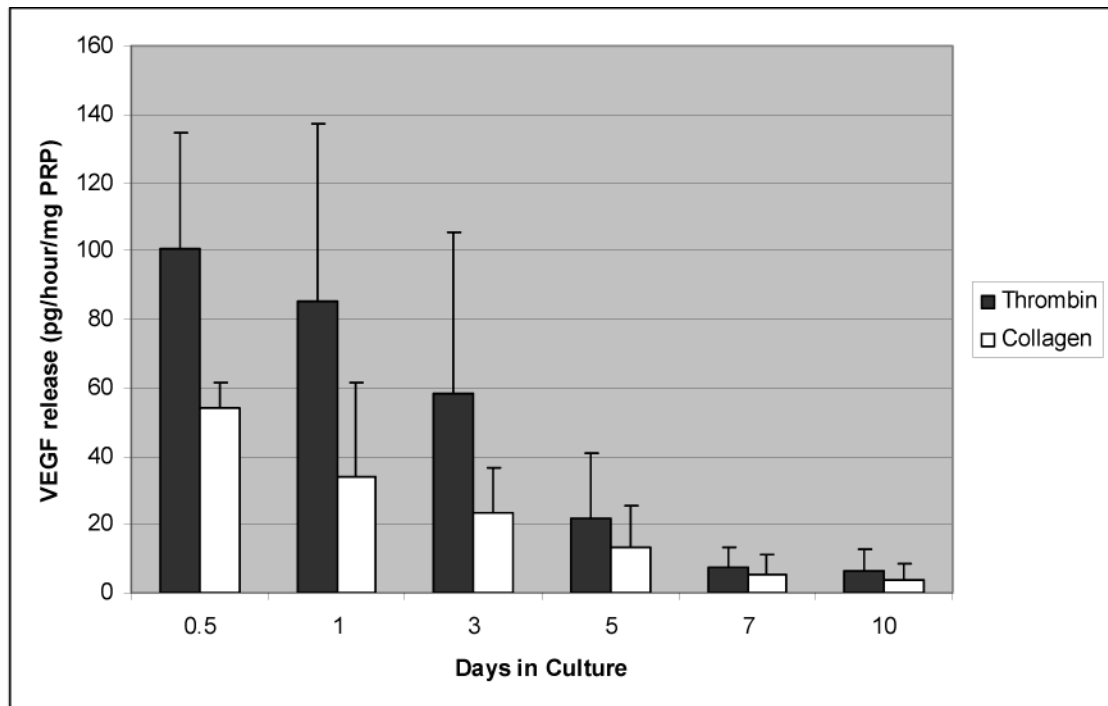


Figure 4. VEGF release in pg/hour from thrombin and collagen PRP clots at time points between 12 hours and 10 days. No significant difference was observed between groups, likely due in part to a relatively large variation in release between patients (repeated measures ANOVA, $p > 0.15$). Points represent the average value at each time point, error bars represent the standard deviation for each group ($n=4$) at each time point.

Table**Whole Blood and PRP Platelet and Granulocyte Counts**

Whole Blood and PRP Platelet and Granulocyte Counts for each of the four donors. Numbers in parentheses indicate the increase over baseline for each of the values in the platelet-rich plasma. Identical platelet-rich plasma samples were used in the thrombin and collagen groups.

Patient #	Platelets		Granulocytes	
	Whole Blood Baseline	PC (times baseline)	Whole Blood Baseline	PC (times baseline)
1	204	860 (4.22)	2.6	1.0 (0.4)
2	194	715 (3.68)	4.8	5.6 (1.2)
3	318	1215 (3.82)	3.3	4.3 (1.3)
4	246	1057 (4.30)	3.3	3.3 (1.0)
Avg	241	962 (4.0)	3.5	3.6(1.0)