Activation of Platelet-Rich Plasma Using Thrombin Receptor Agonist Peptide

Regina Landesberg, DMD, PbD,* Andrea Burke, BS,‡ David Pinsky, MD,‡ Ronald Katz, DMD, PbD,§ Jennifer Vo, MS,‖ Sidney B. Eisig, DDS,¶ and Helen H. Lu, PbD**

Purpose: This study proposes an alternative preparation method of platelet-rich plasma (PRP). Specifically, we compare the use of thrombin receptor agonist peptide-6 (TRAP) and bovine thrombin as a clotting agent in the preparation of PRP.

Materials and Methods: PRP was prepared by centrifugation and clotted with thrombin or TRAP. In vitro clotting times were monitored as a function of TRAP concentration, and clot retraction was determined by measuring clot diameter over time. Following the optimization of TRAP concentration, experiments were repeated with the addition of several commercially available bone substitutes. The release of PRP-relevant growth factors as a function of PRP preparation was also determined.

Results: The most rapid polymerization of PRP takes place with the addition of thrombin, followed by TRAP/Allogro (Ceramed, Lakewood, CO), TRAP/BioGlass (Mo-Sci, Rolla, MN), TRAP/BioOss (Osteohealth, Shirley, NY), and TRAP alone. Thrombin caused considerable clot retraction (43%), whereas TRAP alone resulted in only 15% retraction. TRAP/Allogro, TRAP/BioOss, and TRAP/BioGlass all exhibited minimal retraction (8%).

Conclusions: The use of TRAP to activate clot formation in the preparation of PRP may be a safe alternative to bovine thrombin. It results in an excellent working time and significantly less clot retraction than the currently available methods of PRP production.

© 2005 American Association of Oral and Maxillofacial Surgeons

The use of platelet-rich plasma (PRP) as an adjunct to bone grafting procedures in oral and maxillofacial surgery has seen an increase in popularity since its introduction in 1997 by Whitman et al.1 PRP has technical benefits and theoretically may enhance wound healing by increasing availability of critical growth factors that are released by platelet degranulation.2 Preparation of PRP requires concentrating platelets through centrifugation and subsequent polymerization to form a semisolid gel. Several commercially available methods for enrichment of platelets are currently used in the clinical setting.

At the present, all methods of PRP gelation use calcium and bovine thrombin to initiate PRP clot formation. The use of bovine thrombin has unfortunately been associated with the development of antibodies to clotting factors V and XI and thrombin, resulting in the risk of potential life-threatening coagulopathies.3,4 Consequently, there is a growing interest in identifying alternative agents for PRP clotting.

Thrombin signaling of platelets is mediated by a G protein–coupled protease-activated receptor (PAR). The PAR is activated after thrombin binding...
and subsequent cleavage of the amino-terminal end of the receptor. This new amino terminus acts as a tethered ligand and binds intramolecularly to the body of the PAR, resulting in a transmembrane signal (Fig 1A). In contrast, synthetic peptides such as thrombin receptor agonist peptide-6 (TRAP) activate the receptor independent of receptor cleavage (Fig 1B). TRAP is a hexapeptide that corresponds to amino acids 42 to 47 of the thrombin receptor and mimics the effects of thrombin such as platelet aggregation, an increase in tyrosine phosphorylation, inhibition of cAMP, and increase in cytosolic calcium. These reports suggest that TRAP is a promising candidate as a clotting agent for PRP. The effectiveness of TRAP compared with that of thrombin should be investigated.

After gel formation, PRP undergoes clot retraction, and growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGFβ), and vascular endothelial growth factor (VEGF) are released. The degree of clot retraction could have significant effects on the bioavailability of these growth factors and consequently the clinical efficacy of PRP-enhanced bone regeneration. In addition, excessive shrinkage of the PRP gel may affect graft adaptation, resulting in significant loss of growth factors from the graft composite. The time course and the amount of shrinkage that takes place after PRP gelation using thrombin or alternative clotting agents have not been fully characterized.

Clinically, PRP is routinely combined with bone substitutes such as BioOss, an inorganic bovine bone substitute, AlloGro, demineralized freeze-dried human bone allograft, and 45S5 BioGlass, a melt-derived bioactive glass ceramic, during oral and maxillofacial surgery procedures. BioOss and BioGlass are osteoconductive materials, and Allogro is osteoinductive. The present study will also determine the potential of TRAP-6 to clot PRP in conjunction with bone substitutes. The time course and the amount of shrinkage that takes place after PRP gelation with TRAP in the presence of bone substitutes are also evaluated.

The objective of this study was to investigate the use of TRAP as an alternative to thrombin in the clotting of PRP. The optimal concentration, the time course of gelation, and the resultant clot retraction were evaluated using an in vitro assay system. The hypothesis is that TRAP will offer a safer alternative to PRP gelation resulting in adequate working time and decreased clot retraction compared with thrombin was tested. Furthermore, it was expected that TRAP-initiated PRP clot retraction would result in a reduced release of relevant growth factors, potentially increasing the bioavailability of these regenerative agents.

**Materials and Methods**

**PREPARATION OF PLATELET-RICH PLASMA**

PRP was prepared through a modification of the method of Landesberg et al. Briefly, 60 mL of volunteer blood was collected into 10-mL tubes containing 1.0 mL ACD Solution B (Becton Dickinson, Franklin Lakes, NJ). The ACD solution contained 13.2 g/L trisodium citrate, 4.8 g/L citric acid, and 14.7 g/L dextrose. The tubes were spun at 200 g for 15 minutes. The plasma and buffy coat layer were transferred to 10-mL tubes and spun at 200 g for 10 minutes. The upper half (platelet-poor plasma) was
discarded; the lower (PRP) was resuspended and used for this study.

CLOTTING OF PLATELET-RICH PLASMA WITH THROMBIN AND THROMBIN RECEPTOR AGONIST PEPTIDE-6

PRP (0.5 mL) aliquots were dispensed into 24-well plates precoated with 1% bovine serum albumin (Sigma, St Louis, MO). Thirty microliters of 10% CaCl2 solution (American Reagent Laboratories, Shirley, NY) were added to each well. Experiments were performed in triplicate (n = 3) with the addition of bovine thrombin (30, 100, 300 units; GenTrac, Middleton, WI) or TRAP (10, 50, 100 units; GenTrac, Shirley, NY) were added to each well. Experiments were repeated with the addition of thrombin or TRAP and served as the control group.

Clot retraction was determined by measuring the clot diameter at 1, 2, 4, and 24 hours, and the value was normalized against the well diameter.

PREPARATION OF PLATELET-RICH PLASMA–BONE SUBSTITUTE COMPOSITES WITH THROMBIN RECEPTOR AGONIST PEPTIDE-6

After the optimization of TRAP concentration, experiments were repeated with the addition of 25 or 50 mg of bone substitutes. To minimize nonspecific binding, the culture well (Corning Inc, Corning, NY) was precoated with 1% bovine serum albumin (Sigma) and incubated for 1.5 hours at 37°C. Sterilized BioOss (25 mg, 0.5 to 1.0 mm; Osteohealth, Shirley, NY), AlloGro (25 mg; Ceramed, Lakewood, CO), and 455S Bioactive Glass (25 mg, 300 μm; MoSci, Rolla, MN) were uniformly dispersed within the wells. Fresh PRP (0.5 mL) aliquots were then dispensed into the precoated 24-well plates, and 30 μL of 10% CaCl2 solution (American Reagent Laboratories, Shirley, NY) was added to each well. Experiments were performed in triplicate (n = 3) with the addition of TRAP (H2N-Ser-Phe-Leu-Leu-Arg-Asn-NH2) to the wells containing bone substitutes.

GROWTH FACTOR RELEASE FROM PLATELET-RICH PLASMA PREPARED WITH THROMBIN OR THROMBIN RECEPTOR AGONIST PEPTIDE-6

The temporal release of growth factors was examined as a function of time and mode of PRP preparation. Specifically, the PRP clotted with thrombin or TRAP was allowed to gel, and all samples were incubated at 37°C in a humidified environment for up to 14 days. The volume of fluid released from the clot was measured. Growth factor release was assessed at 1, 3, 7, and 14 days. All collected supernatant samples were stored at −70°C before analysis.

QUANTIFICATION OF PLATELET-DERIVED GROWTH FACTOR AND TRANSFORMING GROWTH FACTOR-β

Supernatants were assayed for PDGF-AB and TGFβ content using diagnostic kits from R & D Systems (Minneapolis, MN). Both assays are based on a sandwich enzyme immunoassay technique. The PDGF-AB assay used a precoated microtiter plate with a monoclonal antibody to PDGF-AA. Preparation and dilution of samples and standards were performed as directed by the manufacturer. Both the standards and the samples were incubated for 3 hours at room temperature. The plate was washed with buffer, and a conjugated antibody to PDGF-BB was added to the wells and incubated at room temperature for 1 additional hour. Following the addition of substrate and termination of the reaction, the absorbance was determined at 450 nm using a spectrophotometer (SPECTRAFluor Plus; Tecan, Maenndorf, Switzerland). A standard curve was generated, and the PDGF-AB level (pg/mL) of each sample was determined. The total amount of growth factors was calculated based on the amount of supernatant obtained after clot retraction.

TGFβ was assayed with a similar enzyme immunoassay technique. A dilution series of TGFβ standards were prepared in 96-well microtiter plates coated with TGFβ receptor Type II. The sample supernatants (0.025 mL) obtained from PRP composites were diluted with 0.075 mL of phosphate-buffered saline solution. The samples were then activated with 0.1 mL of 1.0 N HCl, incubated at room temperature for 10 minutes, and neutralized by an addition of 0.1 mL of 1.2 N NaOH/0.5 mol/L HEPES (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]). The supernatant fractions were then incubated for 3 hours at room temperature. The wells were then washed, and enzyme-conjugated polyclonal antibody to TGFβ1 was added and allowed to incubate for 1.5 hours at room temperature. The reaction was stopped and absorbance was measured at 450 nm using a spectrophotometer (SPECTRAFluor Plus; Tecan). A standard curve was generated, and the TGFβ level (pg/mL) of each sample was determined. The total amount of growth factors was calculated based on the amount of supernatant obtained after clot retraction.

STATISTICAL ANALYSES

All results were expressed as mean ± SD. Multiway analysis of variance (ANOVA) was performed and the Tukey-Kramer test was used to compare between the means. Significance was determined at P < .05.
Results

Thrombin resulted in rapid clotting of PRP. When 30 units of thrombin was added to 0.5 mL of PRP mixture, complete polymerization occurred at 6 minutes. Addition of 100 units (units thrombin/mL PRP used clinically) resulted in clot formation at 3.25 minutes. The addition of thrombin receptor agonist peptide-6 (TRAP) at 100 μmol/L took 9.25 minutes for the clot to completely solidify (Fig 2). The PRP control clot (calcium only) took significantly longer to gel and resulted in a clot with very poor structural integrity (data not shown). Virtually all of the clot retraction in the groups tested was complete by 24 hours. Thrombin caused considerable clot retraction. The addition of 100 units of thrombin led to 43% shrinkage of the clot at all time points, whereas 30 units of thrombin resulted in a 34% clot retraction (Fig 3). In contrast, TRAP at both 50 and 100 μmol/L concentrations showed only a 15% decrease in the clot diameter (Fig 3).

Clotting times using the different bone substitutes were determined using 100 μmol/L of TRAP with 25 mg of Allogro, BioOss, or BioGlass. Clots with Allogro showed complete solidification within 12 minutes, whereas the BioOss (25 mg) composite did not completely polymerize until 13 minutes. The BioGlass group was completely clotted at 8.75 minutes. The clotting time between bone substitutes was not significantly different from each other.

Clot retraction was measured at 2, 24, 72, 168, and 336 hours for all groups with bone substitutes. Retraction was essentially completed by 24 hours, as no significant differences between the 24- and 72-hour measurements were noted for thrombin, TRAP, or TRAP plus bone substitutes. At 24 hours, the TRAP/Allogro (11% ± 2%), TRAP/BioOss (21% ± 4%), and TRAP/BioGlass (8% ± 3%) groups all had significantly less clot retraction than thrombin (56% ± 3%) (Figs 4, 5). Quantitative analyses (Fig 5) of clot diameter corresponded well with observations (Fig 4). In addition, the highest volume of supernatant was collected at day 1. At all time points, the thrombin group measured the largest release volume.

In terms of growth factor release as a function of PRP clotting method, at day 1 the thrombin group released the highest amount of PDGF-AB ($P < .05$, 32,526 ± 6,752.4 pg), approximately 36% more than
At 7 days, the TRAP group had the highest PDGF-AB release (2,797.6 ± 612.0 pg) compared with thrombin (1,738.2 ± 443.0 pg) (Fig 6A). At 14 days, both groups released minimal amounts of growth factor.

The amount of TGFβ released by the PRP composite is presented (Fig 6B). Similar to the case with PDGF-AB, the thrombin group released the highest amount of TGFβ at day 1 postclotting. The total amount of TGFβ contained in the original PRP volume was measured to be 13,982 ± 2,673.81 pg. In the thrombin group, over 81.4% of the growth factor was already released from the clot within 24 hours. In contrast, the TRAP group released significantly lower levels of TGFβ (P < .05). Compared with the thrombin group, clotting of PRP with TRAP retained 39.2% more of the growth factor at 72 hours. After 14 days, all of the TGFβ present in the original PRP thrombin clots had been released.

## Discussion

A number of centrifugation protocols are presently available for concentrating platelets; however, all methods use bovine thrombin to accelerate clot formation. Bovine thrombin has been associated with the formation of antibodies to factors V and XI and thrombin that may result in life-threatening coagulopathies. This study examined the potential of TRAP as an alternative to thrombin for the clotting of PRP. The findings from this study show that compared with thrombin, TRAP preparations of PRP resulted in longer working time, larger clot diameters, and extended bioavailability of specific growth factors necessary for bone regeneration.

TRAP is a synthetic hexapeptide that activates the thrombin receptor independent of receptor cleavage. It corresponds to amino acids 42 to 47 of the thrombin receptor and mimics the effects of thrombin.

An in vitro system was developed here to quantify polymerization time of PRP using thrombin, TRAP, and TRAP-bone substitutes. When a clinically relevant concentration of thrombin was used to clot PRP, it resulted in rapid clot formation with a large amount of clot retraction. In contrast, at concentrations of 50 and 100 μmol/L, TRAP significantly decreased the degree of clot retraction while providing an appropriate working time. The efficacy of TRAP was unaffected with the addition of several bone substitutes (Allogro, BioOss, and BioGlass), as minimal clot retraction was measured when the clot had fully polymerized. In a related study, we have recently shown that on clot retraction, significant levels of growth

---

**FIGURE 4.** Clotting times using the different bone substitutes were determined using 100 μmol/L of thrombin receptor agonist peptide-6 (TRAP) with either 25 mg of Allogro, BioOss, or 4555 Bioactive Glass (BG). Arrows indicate clot size.


**FIGURE 5.** Thrombin receptor agonist peptide-6 (TRAP)/Allogro (11%, 25 mg at 24 hours), TRAP/BioOss (21%, 25 mg at 24 hours), and TRAP/BioGlass (8%, 25 mg at 24 hours) all had significantly less clot retraction than thrombin (56%, 100 units, 24 hours).

Factors are released from the PRP gel. Consequently, decreasing clot retraction in PRP polymerization will potentially retain optimal growth factor amounts with a desired delay in bioavailability as well as maintenance of graft adaptation to the perimeter of the bone defect. Therefore, one of the goals of this study was to identify a PRP clotting agent that would result in minimal clot retraction.

PRP is derived from plasma enriched with platelets and may be efficacious in enhancing bone regeneration when used in oral and maxillofacial surgical procedures. While the use of PRP in bone grafting procedures offers some mechanical advantage based on the adhesive properties of the gel, significant controversy exists regarding the ability of PRP to accelerate bone regeneration. Marx et al performed radiographic and histomorphometric studies on 88 mandibular discontinuity defects of 5 cm or more, where half of the patients received a cancellous posterior ilial bone graft with PRP. The study found that the PRP grafts matured earlier and had higher total bone content than the grafts without PRP. There have, however, been a number of clinical as well as animal studies that have failed to show the efficacy of PRP in facilitating bone repair.

It is postulated that the stimulation of bone healing by PRP is due to the increased concentration of relevant growth factors, including PDGF, TGFβ, vascular endothelial growth factor (VEGF), and insulin-like growth factors (IGFs). While the optimal levels as well as the temporal sequence of growth factor delivery in bone regeneration have not been established, growth factors that promote osteoblast differentiation and maturation (ie, TGFβ and IGF) are believed to act at a later time in the bone regeneration cascade. Within this context, the findings from the present study suggest that the alternate PRP preparation method using TRAP and TRAP/bone substitutes may enhance bone graft integration and maturation by delaying the release of relevant PRP-derived growth factors and extending their bioavailability during the bone regeneration process.

In conclusion, the use of TRAP in the preparation of PRP provides a safe and economical alternative to thrombin while minimizing the amount of clot retraction and the potentially rapid loss of critical bone regenerative growth factors into the interstitium. The development of this in vitro system will allow us to optimize PRP preparation before future clinical trials and ultimately increase its clinical efficacy.

Acknowledgments

The authors wish to acknowledge Dr Rick Tsay and Matthew Cozin for technical support.

References