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Quantification of Growth Factor Levels Using a Simplified Method of Platelet-Rich Plasma Gel Preparation

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Purpose: This study compared two methods of preparing platelet-rich plasma (PRP) gel and the levels of PDGF and TGFβ in each preparation.

Materials and Methods: Platelet-rich plasma gel was prepared by centrifugation and clotted using the ITA gelling agent (Natrex Technologies Inc, Greenville, NC) or by the addition of thrombin and calcium chloride. The levels of platelet-derived growth factor (PDGF) and transforming growth factor beta (TGFβ) generated by clot formation were assayed by enzyme-linked immunoassay (ELISA).

Results: Both methods of preparation yielded PRP gel in less than 30 minutes. However, the ITA preparation did not require thrombin to achieve adequate gel formation. The levels of PDGF and TGFβ were similar regardless of which method was used for initiation of clot formation.

Conclusion: Use of ITA for gel preparation is equivalent to using calcium chloride and thrombin, without the need for special equipment and the risk of coagulopathy.

Platelet-rich plasma (PRP) gel is derived from an autogenous preparation of concentrated platelets. PRP gel has numerous applications, particularly in the cardiac and neurosurgical areas.[1,2] Recently it has undergone a significant increase in use as an adhesive with cancellous bone particles in oral and maxillofacial surgery bone grafting procedures.[3,4]

The traditional method of PRP preparation involves isolating platelets with a cell separator (Medtronics, Parker, CO), followed by gel formation using calcium chloride and bovine thrombin. This procedure has several disadvantages. The equipment necessary is expensive and is generally available only in an operating room or blood bank facility, making the use of PRP in a private office extremely difficult. Furthermore, the use of bovine thrombin has been associated with the development of antibodies to clotting factors V, XI, and thrombin, resulting in the risk of life-threatening coagulopathy.[1,2,5-7] We describe a new method to prepare PRP gel using a simplified armamentarium of equipment and supplies. This procedure can be used in an office setting and will yield an adequate amount of PRP gel for most minor bone grafting procedures. Additionally, this method uses an alternative to thrombin for gelling of the PRP, making it a safer preparation than that currently available.

PRP is known to contain a number of growth factors/cytokines that may aid in the accelerated maturation of a bone graft.[4] Although platelet-derived growth factor (PDGF) and transforming growth factor-β (TGFβ) have been identified in PRP gel, the amounts have not been quantified. It is also unclear as to whether the method used to gel the PRP affects the amount of growth factors/cytokines released from the platelets. We therefore compared the levels of PDGF and TGFβ generated from PRP prepared with the ITA gelling agent (Natrex Technologies Inc, Greenville, NC) and the thrombin/calcium chloride method.

Materials and Methods

PREPARATION OF PRP

Venous blood was obtained from three healthy volunteers (2 males, 1 female). The blood was drawn into 5-mL vacutainer tubes containing either 0.5 mL 0.129 mol/L sodium citrate or 0.048 mL of 15% ethylenediaminetetra-acetic acid (EDTA) (K_3) solution (Becton Dickinson, Franklin Lakes, NJ). An aliquot was removed to determine the platelet count. The tubes were then spun at either 100*g* or 200*g* for 2 to 20 minutes in a Mistral 3000i centrifuge (Sanyo Gallenkamp, Bensenville, IL). All of the plasma was

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transferred to a 15-mL conical polypropylene tube with printed graduations (Corning, Corning, NY) and centrifuged at 100*g*, 200*g*, 250*g*, or 400*g* for 2 to 10 minutes. The upper half of the preparation was designated platelet-poor plasma and the lower half platelet-rich plasma (PRP). Platelet counts were performed on both fractions.

GENERATION OF PRP GEL

The PRP obtained from each donor was aliquoted into two 50-mL glass beakers, and PRP gel was generated using the ITA gelling agent or by addition of thrombin/calcium chloride. For the thrombin/calcium chloride gel, 5 mL of 10% calcium chloride was added to 5,000 units of bovine thrombin (GenTrac, Middleton, WI); 0.167 mL of this mixture was added to each milliliter of PRP and the mixture was allowed to solidify in the beaker at 37°C in a water bath. For the ITA method, 0.06 mL of 10% calcium chloride and 0.5 mL of ITA gelling agent were added to each ml of PRP. The mixture was allowed to gel in a glass beaker at 37°C in a water bath.

QUANTIFICATION OF TGFβ AND PDGF IN PRP PREPARATIONS

The PRP gel preparations prepared above were covered with parafilm and allowed to undergo maximal clot retraction at 4°C overnight. The contents were then transferred to 50 mL polypropylene centrifuge tubes (Corning) and centrifuged at 1,000*g* for 30 minutes. The supernatant amounts were measured and stored at −70°C.

TGFβ1 and PDGF-AB were assayed using diagnostic kits from R & D Systems (Minneapolis, MN). Both assays use a sandwich enzyme immunoassay technique. The TGFβ1 assay uses a microplate which is coated with TGFβ receptor II. Preparation and dilution of samples and standards were performed as directed by the manufacturer. Duplicates of 200 µL aliquots were applied to the microtiter plate, the plate was covered and incubated at room temperature (RT) for 3 hours. The wells were then washed and enzyme conjugated polyclonal antibody to TGFβ1 was added and allowed to incubate at RT for 1.5 hours. The plates were washed, substrate added, and incubated for 20 minutes at RT. 50 µL of stop solution was added and the plates were read on a Dynatech ELISA reader at 450 nm. A standard curve was generated and the TGFβ1 levels (ng/mL) of each sample were determined. The total amount of growth factor was calculated (ng/3 mL PRP) based on the amount of supernatant obtained after clot retraction.

PDGF-AB levels were determined with a similar enzyme immunoassay technique. This assay uses a precoated microtiter plate with a monoclonal antibody to PDGF-AA. The samples and standards were prepared as recommended, applied to the plate and incubated for 2 hours at room temperature. The plate was washed and a conjugated antibody to PDGF-BB was added to the wells and incubated at room temperature for an additional 2 hours. The wells were then washed and substrate was added for 20 minutes at room temperature. The reaction was stopped and read at 450 nm. The concentration (ng/mL) and total yield (ng/3 mL PRP) were calculated as previously described.

Results

Although EDTA consistently gave higher yields than the citrate (approximately 2-fold), the platelets appeared to have been damaged. The EDTA-anticoagulated samples appeared, by light microscopy, to be ragged, and there was considerable noncellular debris present.

The extent of platelet enrichment in the PRP preparations was quantified based on varying the force and time of centrifugation. The results are presented in Tables 1 and 2. Optimal platelet enrichment was achieved with an initial centrifuge spin of 200*g* for 10 minutes (Table 1). The second spin centrifugation of the plasma layer varied from 2 to 10 minutes. Centrifugations of less than 5 minutes failed to achieve any significant platelet enrichment (data not shown). The force of the second spin was therefore evaluated using a 10-minute interval. Both 200*g* and 250*g* enriched the platelets by 200% ($P < .05$). Forces of greater than 250*g* resulted in a platelet pellet that could not be resuspended. Based on these results, all PRP preparations used in subsequent experiments were carried out with both spins at 200*g* for 10 minutes.

The clots formed by the ITA gelling agent and the thrombin/calcium chloride were similar in size and consistency. Representative clot preparations were allowed to gel in a beaker as previously described and were photographed immediately after solidification. The initial PRP gels were similar, although the thrombin/calcium chloride clot began to retract more quickly than the ITA clot.

The results of the TGFβ1 and PDGF-AB assays are shown in Tables 3 and 4. The yield of supernatant was consistently 50% higher in the ITA samples, because of the differences in volume of the clot initiation

Table 1. FIRST SPIN PLATELET YIELDS FROM A REPRESENTATIVE SAMPLE

g Force/Time	Platelets/mL	% Enrichment
Whole blood	2.68×10^8	—
100*g*, 10 min	5.12×10^8	191
200*g*, 10 min	6.15×10^8	229
200*g*, 20 min	4.66×10^8	174

Table 2. SECOND SPIN PLATELET YIELDS

g Force/Time	Platelets/mL (% Enrichment) Patient 1	Patient 2	Patient 3
Whole blood	2.06×10^8	2.94×10^8	1.73×10^8
100*g*, 10 min	3.13×10^8 (52%)	6.85×10^8 (133%)	3.80×10^8 (120%)
200*g*, 10 min	5.57×10^8 (170%)	9.35×10^8 (218%)	5.70×10^8 (229%)
250*g*, 10 min	5.23×10^8 (154%)	8.00×10^8 (172%)	5.70×10^8 (229%)

	Mean % increase ± SE
1. 100/10	101.7 ± 30.7
2. 200/10	205.7 ± 22.2*
3. 250/10	185.0 ± 27.7*

NOTE. Plasma from 1st spin of 200*g*, 10 minutes.
*Significantly different from 100*g*, 10 minutes ($P < .05$).

agents (0.167 mL thrombin/calcium chloride vs 0.560 mL ITA). However, the levels of TGFβ1 and PDGF-AB were similar regardless of the method of preparation.

Conclusions

The use of PRP gel in oral and maxillofacial surgical procedures has technical benefits and may enhance bone regeneration when used in conjunction with autologous bone grafts.[3,4] Production of PRP gel involves the use of an expensive autotransfusion system and solidification of the gel with bovine thrombin. The current armamentarium prevents this procedure from being performed in an office setting. Additionally, the use of bovine thrombin is contraindicated because it may cause life-threatening coagulopathies.[1,2,5-7] The ITA gelling agent was developed to allow PRP gel to be easily prepared in an office setting. This system also offers the advantage of preparing the gel without the use of bovine thrombin.

In 3 separate preparations, 40 mL of donor blood yielded approximately 8 mL of PRP gel. This amount of gel is adequate for most minor surgical procedures, including bilateral sinus grafts, onlay grafts, ridge preservation, and repair of 1- to 2-cm bone defects. Although a laboratory centrifuge was used for preparing the PRP, the low speeds and short times needed

Table 3. TGF-β1 PRODUCTION (TOTAL ng/3 mL PRP GEL)

Sample	Patient 1	Patient 2	Patient 3	Mean ± SE
Plasma	3.89	4.97	7.66	5.51 ± 1.37
Thrombin/Ca^{++}	157.35	113.70	107.36	126.14 ± 19.25
ITA	155.36	127.71	130.30	137.79 ± 10.80

Table 4. PDGF (AB) PRODUCTION (TOTAL ng/3 mL PRP GEL)

Sample	Patient 1	Patient 2	Patient 3	Mean ± SE
Plasma	3.11	2.00	1.88	2.33 ± 0.48
Thrombin/Ca^{++}	118.02	123.88	115.99	119.30 ± 2.90
ITA	111.93	130.94	126.91	123.26 ± 7.08

can be achieved with the inexpensive clinical centrifuge used in most hematology laboratories.

The use of EDTA is potentially more harmful than citrate in the preparation of PRP gel. Although EDTA gave greater yields of platelets, they appeared damaged by the presence of EDTA. The citrate-derived plasma had sufficient platelets to produce good clots.

Healing of an autologous bone graft is a complex biologic cascade in which many of the specific biochemical events that take place have not been well defined. The cancellous bone graft and its associated marrow elements contains many different cell types, each with the potential to manufacture and release specific cytokines and growth factors.[8-10] These cytokines/growth factors most likely play a significant role in the integration and maturation of a bone graft. In both an autologous bone graft and a healing fracture, there is an initial influx of hematopoietic elements from the surrounding healthy bone.[9] Therefore, it is likely that platelets and platelet-derived cytokines/growth factors are present during the early stages of bone graft healing. TGFβ1 and PDGF are known to be produced by platelets and released during degranulation. TGFβ1 has been shown to stimulate proliferation and collagen synthesis by osteoblasts and osteoblast precursors.[11-13] It also may act as a chemotactic agent in recruiting preosteoblasts to the site of bone injury. PDGF also stimulates mitogenesis of osteoblastic precursors.[10,14-17] Although there are numerous cytokines/growth factors that play a role in the specific temporal sequence that occurs during bone graft healing, TGFβ and PDGF most likely contribute to the early influx of cells and stimulation of proliferation.

Our results showed that significant and approximately equal amounts of TGFβ and PDGF were present in the PRP gels, regardless of the method of preparation. The platelet-free plasma was also assayed after removal of all residual platelets. The amounts of TGFβ1 and PDGF present in the platelet-free plasma were minimal (Tables 3 and 4), indicating that almost all of the growth factor/cytokine present in the PRP gel is derived from the platelets.

In these experiments, the quantification of TGFβ1 and PDGF in PRP gel was performed on the serum derived 24 hours after clot formation. It was collected after a hard centrifuge spin to recover all the available fluid from the compacted clot. Although a significant

amount of clot retraction is achieved by 2 hours, maximal retraction takes place by 24 hours.[18] Whereas there may be growth factors/cytokines still sequestered within the remaining clot, enzymatic breakdown by fibrinolysis would be necessary for their release. Our method of assaying the TGFβ1 and PDGF levels allowed the amounts of growth factor that are immediately available to the surrounding bone and tissue surfaces to be quantitated. However, it does not permit a determination of the biologic activity, which can be affected by the method of preparation.

It should be noted that, because some clot retraction takes place immediately, a small amount of liquid appears to remain unincorporated into the gel. We are aware that some practitioners blot the PRP gel before placement in the graft site. Because this fluid has significant amounts of growth factors, we recommend adding it to graft rather than discarding it.

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References

1. Cmolik BL, Spero JA, Magovern GJ, et al: Redo cardiac surgery: Late bleeding complications from topical thrombin-induced factor five deficiency. J Thorac Cardiovasc Surg 105:222, 1993
2. Spero JA: Bovine thrombin-induced inhibitor of factor V and bleeding risk in postoperative neurosurgical patients. Neurosurgery 78:817, 1993
3. Whitman DH, Berry RL, Green DM: Platelet gel: An autologous alternative to fibrin glue with applications in oral and maxillofacial surgery. J Oral Maxillofac Surg 55:1294, 1997
4. Marx RE, Carlson ER, Eichstaidt RM, et al: Platelet-rich plasma: Growth factor enhancement for bone grafts. Oral Surg 85:638, 1998
5. Muntean W, Zenz W, Finding K, et al: Inhibitor to factor V after exposure to fibrin sealant during cardiac surgery in a two-year-old child. Acta Paediatr 83:84, 1994
6. Sosolik RC, Theil KS, Brandt JT: Clinical pathology rounds: Anti-bovine thrombin antibody. Lab Med 27:651, 1995
7. Landesberg R, Moses M, Karpatkin M: Risks of using platelet rich plasma gel (letter). J Oral Maxillofac Surg 56:1116, 1998
8. Linkhart TA, Mohan S, Baylink DJ: Growth factors for bone growth and repair: IGF, TGF beta and BMP. Bone 19:1S, 1996
9. Mundy GR: Regulation of bone formation by bone morphgenetic proteins and other growth factors. Clin Orthop 324:24, 1996
10. Lind M: Growth factor stimulation of bone healing. Effects on osteoblasts, osteomies, and implants fixation. Acta Orthop Scand Suppl 283:2, 1998
11. Horner A, Kemp P, Summers et al: Expression and distribution of transforming growth factor-beta isoforms and their signaling receptors in growing human bone. Bone 23:95, 1998
12. Duneas N, Crooks J, Ripamonti U: Transforming growth factor-beta 1: Induction of bone morphogenetic protein genes expression during endochondral bone formation in the baboon, and synergistic interaction with osteogenic protein- (BMP-7). Growth Factors 15:259, 1998
13. Ripamonti U, Duneas N, Van Den Heever B, et al: Recombinant transforming growth factor-beta 1 induces endochondral bone in the baboon and synergizes with recombinant osteogenic protein-1 (bone morphogenetic protein-7) to initiate rapid bone formation. J Bone Miner Res 12:1584, 1997
14. Sandy J, Davies M, Prime S, et al: Signal pathways that transduce growth factor-stimulated mitogenesis in bone cells. Bone 23:17, 1998
15. Hsieh SC, Graves DT: Pulse application of platelet-derived growth factor enhances formation of a mineralizing matrix while continuous application is inhibitory. J Cell Biochem 69:16, 1998
16. Yu X, Hsieh SC, Bao W, et al: Temporal expression of PDGF receptors and PDGF regulatory effects on osteoblastic cells in mineralizing cultures. Am J Physiol 272:C1709, 1997
17. Horner A, Bord S, Kemp P, et al: Distribution of platelet-derived growth factor (PDGF): A chain mRNA, protein, and PDGF-alpha receptor in rapidly forming human bone. Bone 19:353, 1996
18. Miale JB: Laboratory Medicine Hematology. St Louis, MO, Mosby, 915. 1982, p 915