The use of platelet-rich plasma (PRP) has become more generally accepted, and implant dentists are using PRP more frequently to promote the healing of oral surgical and/or periodontal wounds. Critical elements of PRP are thought to be growth factors contained within the concentrated platelets. These growth factors are known to promote soft-tissue healing, angiogenesis and osteogenesis. We present a rapid, simple, and inexpensive methodology for preparing PRP using the Cliniseal centrifuge method. This study demonstrates that platelets are concentrated approximately 6-fold without altering platelet morphology. Further we demonstrate that key growth factors, platelet-derived growth factor BB (PDGF-BB), transforming growth factor B (TGF-B1), vasculature endothelial growth factor (VEGF), and epidermal growth factor (EGF) are present in comparable or higher concentrations than those reported with the use of other techniques. Prolonged bench set time (>3 hours) after centrifugation resulted in decreased concentration of TGF-B1 but not decreased concentration of PDGF-BB, VEGF, or EGF. This study confirms the molecular aspects of PRP obtained using this inexpensive and efficient methodology.

Key Words: platelet-rich plasma, PRP, growth factors, wound healing
Responds. Activation of GF receptors may stimulate cell growth and differentiation, angiogenesis, apoptosis, or production of extracellular matrix components. In addition, PRP contains white blood cells (WBCs) that may be instrumental in wound healing, particularly in preventing infection.

Oral surgical and periodontal wound healing involves cells at the surgical site, including gingival fibroblasts, gingival epithelial cells, periodontal ligament fibroblasts, and osteoblasts, all of which are important in soft-tissue and hard-tissue repair. 

Topical application of GFs to these cells has been demonstrated to promote the healing of surgical wounds in animals. Review of the medical and dental literature supports the use of autogenous PRP in clinical practice. PRP was approved for clinical use in 1998; it is used for dental implant procedures, bone grafting, and periodontal surgeries and is applied directly into extraction sockets to facilitate healing. Marx et al reported the first clinical dental results of PRP in 1998. These investigators used PRP to improve cancellous bone marrow graft integration for mandible reconstruction after tumor removal. The data demonstrated strong evidence for an accelerated rate and degree of bone formation.

In a 1999 study by Anitua, extraction sites treated with a combination of autogenous bone and PRP demonstrated better epithelialization and more compact mature bone with well-organized trabeculae than did the group receiving autogenous bone alone. Kassolis et al used PRP with freeze-dried bone allograft particles and no evidence of inflammatory cell infiltration.

In 2002, a controlled clinical trial compared the combination of the xenograft bovine porous bone mineral (BPBM) and PRP versus BPBM alone and PRP alone. Six-month postoperative evaluations revealed superior regenerative effects in the PRP-treated groups.

Anecdotal clinical reports by practitioners who routinely use PRP for implant surgeries, extractions, and periodontal surgeries report a faster soft-tissue healing response plus fewer postoperative complications, such as dehiscence, dry sockets, or bleeding. In a retrospective study of more than 900 third molar extractions, with or without PRP, we have previously demonstrated that PRP dramatically reduced the risk of alveolar osteitis (dry socket).

Several published reports also question the effectiveness of PRP. Variations in effectiveness may be due to differences in methods of PRP preparation. The mechanism for GF release, receptor binding, and resulting nuclear response is complex and therefore may be compromised or enhanced by small differences in platelet concentration/enrichment and PRP delivery techniques. In a clinical office setting, dental personnel can use a number of procedures to concentrate platelets to produce PRP for immediate postoperative use. Techniques that ensure the production of large quantities of autologous PRP can be time-intensive, labor-intensive, and expensive. In contrast, techniques that produce small quantities of PRP can be time-efficient, cost-efficient, and easily accomplished by dental auxiliaries. Four of the better-known commercial systems available for dental office use include S Mart-PRP autologous platelet-concentrate system (Harvest Technologies Corporation, Plymouth, Mass), Platelet Concentrate Collection System (PCCS; 3i Implant Innovations, Palm Beach Gardens, Fla), Curasan PRP Kit (Curasan, Pharma Gmbh AG, Lindigstrab, Germany), and Ace Platelet Concentration System (Ace Surgical Supply Company, Brockton, Mass). These commercial systems have all undergone some form of testing to determine platelet concentration and GF availability.

A variety of other conventional systems are available for use by dental practitioners that involve the basic protocols for blood collection, platelet concentration via centrifugation, and delivery to the surgical site via a syringe. One such generic system uses the Clinaseal Centrifuge (Salvin Dental Specialties, Charlotte, NC). This is an inexpensive single-speed bench-top centrifuge that is used by many dentists. Use of the Clinaseal Centrifuge is a simple and cost-effective method of concentrating platelets and the desired GFs contained within them. Although Eby reported an average platelet yield increase of 356% versus whole blood using this method, there is only limited analysis of the use of this centrifuge with a definitive protocol and of its effectiveness in concentrating intact platelets and confirming the presence of GFs.

In this article we investigate the use of the Clinaseal Centrifuge to obtain PRP and examine platelet enrichment as a function of centrifugation time. We also examine the effect of bench-setting time on PRP platelet enrichment, platelet microscopic morphology, and GF content (PDGF-AA, PDGF-BB, ILGF, NGF, EGF, VEGF, and TGF-β1) using Western blotting and enzyme-linked immunosorbent assays (ELISAs).

### Materials and Methods

#### Collection of PRP

All blood collection methods were approved by the Duquesne University Institutional Review Board. Sub-
ject recruitment was from the author’s private practice (J.L.R.). All 7 recruited subjects participated in the study. Whole blood was drawn using a 21-gauge 1.5-inch latex-free needle (Exelint International Co, Los Angeles, Calif) and 4.5-mL BD Vacutainer tubes (Becton Dickinson & Co, Franklin Lakes, NJ) containing 0.45 mL of the anticoagulant trisodium citrate (9:1). For each experiment, one Vacutainer tube was used to acquire a baseline whole blood platelet value. The remaining BD Vacutainer tubes were centrifuged for various times at 1350g using the Clinaseal Centrifuge to assess the optimal spin time for recovering platelets in PRP.

After centrifugation, the tubes were removed and placed in a test tube rack on the bench for various time intervals to ascertain whether bench set time affected the recovery of platelets in PRP. The red blood cell (RBC)/plasma interface was allowed to set for 3 minutes. Premade labels were placed on the outside of the tube to reproducibly delineate the PRP layer to be harvested. Labels possessed a center dotted line positioned at the blood cell/plasma interface, a solid line 3 mm above the dotted line, and a second solid line 2 mm below the dotted line (Figure 1). The upper plasma layer (platelet poor plasma, PPP) was aspirated to the 3-mm mark (top solid line) using a sterile, aluminum, blunt-end, 20-gauge 1.5-inch needle (Monoject No. 8881202363, Tyco Healthcare Group LP, Mansfield, Mass) attached to a 3-mL B-D latex-free syringe (Becton Dickinson & Co). The PRP was collected from between the upper 3-mm mark and the lower 2-mm mark using a beveled 20-gauge 1-inch B-D Precision Glide needle (Becton Dickinson & Co) attached to a 3-mL B-D latex-free syringe.

Platelet concentration determination
Platelets were counted using a Cell Dyn 1600 cell counter (Abbott Laboratories, Abbott Park, Ill). Because of the viscosity of PRP it was necessary to dilute the PRP 1:10 with Streck Diluent (Streck Laboratories, Omaha, Neb) before introducing the sample into the cell counter. The diluted sample was gently mixed by inversion to obtain a uniform suspension before aspiration and analysis using the cell counter.

Platelet morphology in PRP
Smears of PRP were stained with Wright-Giemsa stain and then examined by oil immersion brightfield microscopy at ×1000 magnification using a Nikon Eclipse 600 microscope (Fryer Co Inc, Huntley, Ill). Several fields were examined for each sample, and representative photomicrographs were obtained using the SPOT RT-Slider camera and SPOT Advanced Digital Image Analysis software (Fryer Co Inc).

Partial purification of platelets from PRP
To remove RBCs from PRP, the Histopaque 1077 cell separation medium (Sigma-Aldrich, St Louis, Mo) was used. Briefly, PRP was diluted 10-fold with Hank’s
Balanced Salt Solution (HBSS; Mediatech, Herndon, Va) and overlaid onto 5-mL Histopaque 1077 density gradient medium (Sigma-Aldrich, St Louis, Mo). The overlaid PRP was then centrifuged at 900g for 20 minutes. The band containing platelets, lymphocytes, and monocytes was aspirated using a sterile polypropylene pipette, diluted with HBSS, and then centrifuged for 20 minutes at 900g. Platelet-rich pellets were then resuspended and washed 2 times in HBSS for 5 minutes at 900g; platelet counts were performed as before. To lyse platelets, the washed pellets were suspended in 0.5% Triton X100 and gently rocked on an Ocelot shaking platform (Fisher Scientific, Sigma-Aldrich) for 40 to 60 minutes. Lysis was verified by microscopic examination and by subjecting the lysate to centrifugation as before, with no visible pellet observed. The lysate was then used for analysis by immunoblotting to indicate the presence of specific GFs, or by ELISA to quantify specific GFs.

**Western blotting**

Precast 5% to 20% polyacrylamide gradient gel wells (Jule Biotechnologies, Milford, Conn) were loaded with either Kaleidoscope molecular weight standards (Bio-Rad, Chicago, Ill), positive-control purified human recombinant GFs (US Biologicals, Swampscott, Mass), or partially purified PRP samples. The samples were subjected to electrophoresis for approximately 75 minutes at 150 V, 35 mA, and 8 W. Transfer of proteins to nitrocellulose paper (BioRad) was performed at 100 V at 3°C for 1 hour. Blots were then placed in Tris-buffered saline (TBS; 20 mmol/L Tris, 150 mmol/L NaCl) with 0.5% vol/vol Tween 20 (TBST) and 3% bovine serum albumin to block nonspecific binding for 8 hours or overnight.

The blots were then removed and washed 3 times with TBST and placed in a sealed storage bag with the appropriate primary antibody (US Biologicals) at the manufacturer’s specified concentration for Western blotting (20 ng/mL TGF-β1, 0.2 μg/mL PDGF, 0.2 μg/mL NGF) in TBST for 4 hours at 3°C. The blots were removed and washed 3 times with TBST, and then placed in anti-rabbit IgG alkaline phosphatase conjugate (US Biologicals) as the secondary antibody for 1 hour. The blots were then removed, washed 3 times with distilled water, and placed in a solution of 5-bromo-4-chloro-3-indolyl phosphate nitro blue tetrazolium (Sigma Fast Tablets, Sigma-Aldrich). Color development was allowed to proceed until positive-control GF bands were readily apparent, and then it was stopped by immersing the blot in distilled water. The blots were then photographed using a Sony Digital Mavica camera (Sony Corporation, New York, NY).

**ELISAs**

We used ELISAs to determine the quantity of various GFs. The ELISA data are from one patient over different days and time points. Lysed platelet samples were prepared for ELISA with Quantikine kits (R&D Systems, Minneapolis, Minn). The manufacturer’s protocol was followed with the following modifications. To fully release TGF-β, it was necessary to use a 3-fold higher volume (600 μL) of 1 N HCl with incubation of lysates at room temperature for the specified time. This change required the lysate to be subsequently neutralized using 600 μL 1.2 N sodium hydroxide. It was also necessary to dilute samples 10-fold before adding to the anti-TFG-β1-coated wells because the residual 0.5% Triton X-100 used for lysis interfered with the ability of TGF-β1 to bind to the wells. The PDGF-BB samples were diluted 5-fold to ensure that the absorbance was in the range of the standard curve. After color development, quantification was performed using a BIO-TEK Kinetic Microplate Reader Model EL312E (MTX Lab Systems, Inc, Vienna, Va).

**Statistical analysis**

Statistical analysis was performed using SPSS 15.0 (SPSS, Inc, Chicago, Ill). A 1-way analysis of variance was performed on the data obtained from quantifying the GFs (ELISAs) as shown in the Table.

**RESULTS**

The first objective was to determine the optimum time of centrifugation in the Clinaseal Centrifuge to obtain the most consistent and greatest enrichment of the PRP. The enrichment of platelets in PRP over whole blood using the Clinaseal Centrifuge is displayed in Figure 2. A greater than 6-fold enrichment of platelet concentration was obtained consistently using 10 minutes of centrifugation. For this reason, the 10-minute centrifugation time was chosen as the optimal time and used for the duration of the study.

The next objective was to determine whether bench set time (time between blood draw and application in the patient) affected the recovery and enrichment of platelets in PRP. Figure 3 shows the enrichment of platelets as a function of bench set time. No significant differences were found; however, the SDs varied and were found to be much larger as bench set time increased. While the number and concentration of platelets do not appear to change as
a function of bench set time, platelets in PRP may change in other ways and thereby become less active in promoting healing. The third objective was to determine whether the morphology of platelets changes as a function of bench set time. Figure 4A shows the components of a typical stained whole blood smear. Figure 4 shows the changes that occurred in platelet morphology from 0 to 6 hours bench set time. From 0 to 2 hours, platelets appear normal, much like they appear in whole blood: discoid and lightly stained with the blue Giemsa dye. The platelets displayed little to no aggregation. However, after 2 hours of bench set time, platelets began to appear smaller and more densely stained and formed small clusters. These effects were more dramatically evident by 6 hours, indicating a change in their morphology as a function of bench set time.

The effectiveness of PRP in wound healing and other responses is thought to be due to the presence of GFs in the platelets. Western blots shown in Figure 5 demonstrated that the PRP obtained contains GFs including PDGF-BB, TGF-β, and NGF. Since GFs are small proteins (5 to 30 kd/atomic mass units), and their structure and activity may be sensitive to hydrolytic or proteolytic degradation over time, the next objective was to investigate whether the platelet morphologic changes that occur with increasing bench set time were accompanied by changes in GF content of the PRP. We used ELISAs to measure the content of TGF-β1, EGF, VEGF, and PDGF-BB in PRP as a function of bench set time.

| Table |
|-----------------
| Growth Factor Content* (pg/10⁶ platelets) | Bench Set Time (hours) |
| | 0 | 3 | 6 |
| | Mean ± SD | Range | Mean ± SD | Range | Mean ± SD | Range |
| TGF-β1 | 485 ± 138 | 311–751 | 382 ± 127 | 201–599 | 432 ± 192 | 256–761 |
| PDGF BB | 3.1 ± 0.98 | 1.06–4.33 | 2.58 ± 0.76 | 1.96–4.1 | 2.9 ± 0.46 | 2.58–3.79 |
| EGF | 0.21 ± 0.19 | 0.03–0.46 | 0.031 ± 0.25 | 0.06–0.68 | 0.333 ± 0.31 | 0.02–0.78 |
| VEGF | 2.08 ± 0.90 | 1.4–2.2 | 2.48 ± 1.01 | 1.81–2.32 | 2.62 ± 1.58 | 1.5–2.4 |

*TGF-β1 indicates transforming growth factor-β1 (n = 9); PDGF BB, platelet-derived growth factor-BB isoform (n = 9); EGF, epidermal growth factor (n = 9); VEGF, vascular endothelial growth factor (n = 6).
FIGURE 4. The morphology of platelets in platelet-rich plasma (PRP) change as a function of bench set time. Centrifuged blood was allowed to set on the bench for up to 6 hours, and then PRP was harvested at various times. Samples were smeared, stained with Wright-Giemsa, and viewed/photographed using brightfield microscopy. All fields are ×1000 magnification. (A) Whole blood; note that very few platelets are present relative to the number of red blood cells (RBCs). (B) A 0 bench set time; note that the platelets are higher in number relative to the RBCs, indicating that platelets have been enriched using the procedures described previously. Also note that the platelets possess a normal morphology (discoid, light blue staining, with little to no adherence to one another [aggregation]). (C) A 30-minute bench set time; concentrated platelets continue to appear intact and healthy. (D) A 1-hour bench set time; platelets begin to aggregate but they are still ovoid and lightly stained. (E) A 2-hour bench set time; platelets form larger aggregates and have begun to lose ovoid shape developing bleb-like structures on the plasma membranes. (F) A 6-hour bench set time; note the aggregation occurring with prolonged bench set time with more than 100 platelets per aggregate. Platelets appear shrunken and misshapen, suggesting a loss of functional capacity.
Because of the prohibitive cost and time required for completing ELISAs, the data are based on PRP obtained from one patient. Baseline ELISAs were completed to determine that one patient could be used as a representative sample (data not shown). The Table shows that levels of these GFs were comparable to the levels reported by others using more complicated techniques of PRP preparation. Although some variation was observed in the data collected, the effect of bench set time did not have a significant effect on the amount of GFs obtained from the platelet lysates. These data seem to indicate that platelets, when not activated, retain significant GF concentration over time.

**DISCUSSION**

The goal of this study was to determine whether a simplified, inexpensive procedure to obtain PRP would yield intact platelet and GF in concentrations similar to those reported in the literature to facilitate clinical healing. The method used readily available vacutainers with citrate as the anticoagulant; needles and syringes that are convenient, inexpensive, and commonly used in clinical practice; and a small bench-top centrifuge with one set speed. This technique makes the entire process user friendly; thus, errors and training time are kept to a minimum. The process takes less than 30 minutes from the start of blood draw to administration in the patient.

This investigation showed that platelet recovery and enrichment in PRP prepared using the Cliniseal Centrifuge was optimal and consistent after 10 minutes of centrifugation (Figure 2). It was also shown that platelet recovery and enrichment did not change significantly after centrifugation for up to 6 hours at room temperature (Figure 3). Thus, this method of PRP preparation provides platelet concentrations and recoveries that match or exceed the PRP preparations obtained through more expensive and elaborate methods.\(^{23,24}\) Also of interest are the WBCs present in PRP preparations. A maximal concentration was obtained at 10 minutes in addition to the platelets (data not shown), which is further evidence to suggest that the 10-minute spin is superior. However, it was found that platelet morphology did change over a period of 6 hours bench set time (Figure 4). Even with 2 hours of bench set time, platelets in PRP began to appear less normal and, as an example, small aggregates formed that are indicative of unwanted platelet activation and GF release. In Western blots at 0 hour bench set time, detectable levels of the GFs PDGF-BB, TGF-β1, and NGF were present in partially purified platelets using this method of PRP preparation. ELISA and statistical analysis demonstrated that PDGF-BB, EGF, and VEGF levels do not significantly change over 6 hours of bench set time. Although the amount of TGF-β1 appears to decrease, it is not a significant decrease (the Table). A larger sample size might have clarified a potential relationship between TGF-β1 concentration and time. Since TGF-β1 is known to stimulate osteogenesis,\(^{10,15,26}\) a loss or decrease in the bioactivity of this factor in PRP preparations may account for the disparity among clinicians concerning the effectiveness of PRP use. The current results suggest that PRP bench set times should not exceed 2 hours to maintain maximal levels of TGFβ1 and of platelet morphology.

There are a variety of explanations for why the time between blood draw and PRP harvest might exceed 2 hours. One obvious reason is that different methods of PRP collection vary in the length of time required to obtain the PRP for use; another is that if the blood is drawn from the patient on arrival at the practice, some simple dental surgical procedures may require less than 30 minutes until PRP application whereas other more complex surgeries may require more than 3 to 4 hours. In practice, it is easiest to draw blood from the patient initially, centrifuge the

![Figure 5. Platelets in platelet-rich plasma (PRP) prepared using the Cliniseal Centrifuge method contain intact growth factors. Western blotting was used to qualitatively assess whether platelets in PRP prepared using the methods described contain platelet-derived growth factor (PDGF-BB) or transforming growth factor-β1 (TGFβ1). Lane 1: molecular weight markers that flank the growth factor protein bands of interest. Lane 2: purified recombinant human growth factors that serve as positive controls. Lane 3: lysate from 5 to 7 × 10^6 partially purified platelets contained within the PRP. Note the presence of bands in Lane 3 that correspond in migration and immunoreactivity to the standard growth factors in Lane 2. Also note that the bands in Lanes 2 and 3 migrate to the known molecular weight range for each growth factor monomer (PDGF-BB = 15 kD, TGFβ1 = 12.5 kD, nerve growth factor = 14 kD).](http://meridian.allenpress.com/joi/article-pdf/34/1/25/2034566/1548-1336(2008)34[25_aaoars_0_co_2.pdf)
vacutainer immediately after draw, but then harvest the PRP layer just before administration. The centrifuged blood therefore will vary in the length of time it remains on the bench top before PRP is collected and used. The results suggest that bench set time influences the activity of PRP and its use for positive clinical outcomes.

For implant dentists, bone formation facilitated by the use of PRP is a crucial part of PRP’s efficacy. A decrease in TGF-β1 concentration in PRP preparation has been noted in platelet and PRP preparations of others. The loss of this factor over time could be attributable to 3 confounding factors, including (1) natural hydrolysis, (2) degradation by proteases, or (3) specific irreversible binding by molecules that may be present in various preparations of PRP. TGF-β1 is normally found in a latent form within platelets. When TGF-β1 is in the latent form it is either bound to latency-associated peptide (LAP) or latent TGF-β binding protein (LTBP), and its release from these proteins is required before it can bind to receptors on target stem cells to trigger the formation of osteoblasts and thus form new bone tissue. If TGF-β1 is released from LAP or LTBP before reaching its receptor, it is rapidly degraded by hydrolysis. Therefore, hydrolysis is one explanation for the loss of TGF-β1 over time in the PRP preparations.

Another explanation for the loss of TGF-β1 is through the action of proteases. If thrombin and Ca2+ are used in conjunction with PRP, the clotting cascade begins and platelets are activated to secrete a variety of granule components along with their GFs. Two types of proteases found within platelet granules are matrix-metalloproteinase-2 (MMP-2) and thrombospondins 1 and 2 (TSP1, TSP2). One effect of these proteases is the release of TGF-β1 from its bound form, rendering it active. The MMP-2 is found as a proteolytically cleave its substrates. Activation of MMP-2 depends on other enzymes (MMP-14 and 16) and because forming a PRP gel by inducing clot formation with Ca2+ and thrombin appears to decrease the amount of TGF-β1 in PRP preparations, the clinical effects of PRP application with and without thrombin and Ca2+ over time needs to be investigated. Future plans include experiments assessing the neutralizing activity of α2M by combining PRP with PPP in various quantities to examine whether immunoprecipitation of α2M from PPP allows higher levels of TGF-β1 to persist in PRP. Future studies will also address the in vitro efficacy of PRP on mesenchymal stem cell differentiation into osteoblasts.
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