Platelet-Poor and Platelet-Rich Plasma Stimulate Bone Lineage Differentiation in Periodontal Ligament Stem Cells

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Background: Plasma-derived fractions have been used as an autologous source of growth factors; however, limited knowledge concerning their biologic effects has hampered their clinical application. In this study, the authors analyze the content and specific effect of both platelet-rich plasma (PRP) and platelet-poor plasma (PPP) on osteoblastic differentiation using primary cultures of human periodontal ligament stem cells (HPLSCs).

Methods: The authors evaluated the growth factor content of PRP and PPP using a proteome profiler array and enzyme-linked immunosorbent assay. HPLSCs were characterized by flow cytometry and differentiation assays. The effect of PRP and PPP on HPLSC bone differentiation was analyzed by quantifying calcium deposition after 14 and 21 days of treatment.

Results: Albeit at different concentrations, the two fractions had similar profiles of growth factors, the most representative being platelet-derived growth factor (PDGF) isoforms (PDGF-AA, -BB, and -AB), insulin-like growth factor binding protein (IGFBP)-2, and IGFBP-6. Both formulations exerted a comparable stimulus on osteoblastic differentiation even at low doses (2.5%), increasing calcium deposits in HPLSCs.

Conclusions: PRP and PPP showed a similar protein profile and exerted comparable effects on bone differentiation. Further studies are needed to characterize and compare the effects of PPP and PRP on bone healing in vivo. J Periodontol 2016;87:e18-e26.

KEY WORDS
Calcification, physiologic; cell differentiation; mesenchymal stromal cells; periodontal ligament; platelet-rich plasma; regeneration.

Several regenerative approaches have been proposed for the treatment of periodontal lesions, including the use of recombinant growth factors that may stimulate signaling cascades in a wide range of cells, promoting proliferation, differentiation, and cell migration.¹² These responses may include the recruitment of stem or progenitor cells to the damaged area to achieve periodontal regeneration.¹³-⁷ In recent years, human blood-derived fractions have been used to improve wound healing as an autologous source of growth factors, using platelets as therapeutic tools.⁸-¹³ Platelets contribute to hemostasis by preventing blood loss at sites of vascular injury, since their α-granules contain growth factors and cytokines that play a key role in inflammation and tissue repair.¹³-¹⁵ Distinct platelet-derived fractions have been designed.¹⁴,¹⁶ These include platelet-rich plasma (PRP), platelet-poor plasma (PPP), and platelet-rich fibrin, among others.¹⁶,¹⁷ At the clinical level, they may be applied in the form of a gel on surgical wounds as an autologous source of biomolecules for the treatment of a wide range of tissue injuries.¹³,¹⁷-¹⁹ However, conflicting results regarding the clinical effectiveness of PRP in periodontal regeneration have been reported in the literature, probably because of wide variations in the protocols used to generate PRP, heterogeneity among clinical studies that evaluate different types of defects, and the absence of a biologic rationale to define the

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adequate concentrations of growth factors and other components present in platelet-derived fractions.\textsuperscript{18-23} PPP, the blood fraction with reduced counts of platelets, appears to promote wound healing–associated cell function and improves the migration and proliferation of gingival fibroblasts in vitro.\textsuperscript{8,9,24} Recently, a clinical study demonstrated similar results for PRP and PPP in intrabony periodontal defects in humans.\textsuperscript{25} Similarly, a study in dogs showed a positive effect on bone healing of extraction sockets in dogs.\textsuperscript{26} Given the studies above, there is a need to characterize the composition and biologic effects exerted by PRP and PPP. In the present study, the authors evaluate and compare the growth factor composition of PRP and PPP obtained from the same donors and assay the effects of these formulations on the differentiation potential of human periodontal ligament stem cells (HPLSCs) to the osteoblastic phenotype.

**MATERIALS AND METHODS**

**HPLSC Cultures**

Healthy human impacted third molars were collected from four young adult females, aged 18 to 24 years, under approved guidelines set by the Ethical Committee of the Pontifical Catholic University of Chile (Pontificia Universidad Católica de Chile [12-117]), Santiago, Chile. Written informed consent was obtained from all donors. Explants, obtained from the middle third of the root, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum (FBS) and a mix of antibiotics (100 U/mL penicillin, 100 \( \mu \)g/mL streptomycin) and antimicrotics (0.25 \( \mu \)g/mL amphotericin B), according to a previously described protocol.\textsuperscript{27} All HPLSC cultures were used in passages 2 to 5.

**Flow Cytometry**

Cells were characterized according to the International Society for Cellular Therapy’s minimal criteria for defining mesenchymal stem cells.\textsuperscript{28} Cells were analyzed by flow cytometry for positive mesenchymal stem cell markers with antibodies against cluster of differentiation 73 (CD73)-V450,\textsuperscript{§} peridinin chlorophyll and cyanine 5.5–conjugated CD105,\textsuperscript{‖} and phycoerythrin and cyanine 7–conjugated CD90.\textsuperscript{¶} The authors also analyzed the presence of negative markers, using antibodies to detect CD11b, CD79a, CD14, CD45, and CD34 coupled with phycoerythrin.\textsuperscript{∥} After antibody incubation, cells were sorted in a flow cytometer\textsuperscript{**} with all antibodies in the same sample.\textsuperscript{29}

**Immunofluorescence**

Cells were grown on coverslips, fixed with paraformaldehyde 4%, blocked, and incubated with primary antibodies overnight. Primary antibodies used were mouse monoclonal anti-STRO-1\textsuperscript{††} (1:20) and rabbit monoclonal anti-CD146\textsuperscript{†††} (1:50). Subsequently, cells were incubated with appropriate secondary antibodies\textsuperscript{§§} (1:400) and counterstained with 4’,6-diamidino-2-phenylindole as previously reported.\textsuperscript{30}

**Differentiation Potential of HPLSCs**

The ability to differentiate into adipose, cartilage, or bone/cementum lineages was evaluated by treating cells for 21 days with specific culture media: adipogenic media (DMEM supplemented with FBS 10% and adipogenic factors [1 \( \mu \)M dexamethasone, 100 \( \mu \)M indomethacin, 0.5 mM isobutylmethylxanthine, 10 \( \mu \)g/mL insulin]); chondrogenic media;\textsuperscript{‖‖} or osteogenic media (DMEM, FBS 10%, 0.1 \( \mu \)M dexamethasone, 10 mM \( \beta \)-glycerophosphate, 50 \( \mu \)g/mL ascorbic acid 2-phosphate). Medium was changed every 2 to 3 days. After incubation, cells were fixed with buffered formalin and stained for cartilage (alcan blue), adipose tissue (oil red O), or bone/cementum tissue (alizarin red) as previously reported.\textsuperscript{29,31}

**PRP and PPP Collection**

PRP and PPP were obtained from four healthy male volunteers, aged 20 to 22 years, using a kit\textsuperscript{¶¶} after obtaining written informed consent from the donors. PRP was prepared from 60-mL blood samples following the manufacturer’s instructions. Blood samples were centrifuged at 3,200 rpm for 15 minutes. To induce platelet activation, PPP and PRP fractions were incubated for 1 hour at 37°C with 1% CaCl\textsubscript{2} and autologous thrombin (10% of final volume) obtained from each patient. After activation, both PPP and PRP were agitated in a vortex for 1 minute and centrifuged for 10 minutes at 3,200 rpm. Platelet-release supernatants were filtered, aliquoted, frozen, and maintained at –80°C until experiments were performed.\textsuperscript{9}

**Proteome Profiler Array Studies**

Both PRP and PPP were evaluated using a semi-quantitative, sandwich-based proteome profile array\textsuperscript{##} including 44 proteins. Briefly, plasma fractions were incubated according to the manufacturer’s instructions. Spots were detected by enhanced chemiluminescence and quantified by densitometry analysis, with respect to internal positive controls, using software.\textsuperscript{***32}

**Growth Factor Quantification**

PRP and PPP were analyzed using enzyme-linked immunosorbent assay (ELISA) kits to quantify the following growth factors: platelet-derived growth factor (PDGF)-BB, PDGF-AA, PDGF-AB, fibroblast growth...
factor (FGF)-2, and epidermal growth factor (EGF).††† Briefly, samples were incubated according to the manufacturer’s instructions and quantified by absorbance at 450 nm, including a standard curve.

**PPP and PRP Treatments**

Cells were grown in DMEM supplemented with 10% FBS on 2-cm² cell culture vessels at 1.5 x 10⁵ cells/mL or on a 96-well plate at 3.2 x 10⁴ cells/mL. At 80% confluence, cells were incubated with DMEM plus PPP or PRP (2.5%, 5%, or 10%) and supplemented with osteogenic factors (0.1 µM dexamethasone, 10 mM β-glycerophosphate, 50 µg/mL ascorbic acid 2-phosphate). Quantities of DMEM supplemented with FBS (2.5%, 5%, or 10%) with or without osteogenic factors were used as controls. Medium was changed every 2 days. At the same time, the Saos-2 cell line‡‡‡ from human osteosarcoma was used as a control. After 14 or 21 days of culture, cells were incubated overnight with 2N HCl to extract calcium deposits. The calcium ions in each sample were quantitated by the o-cresolphthalein complexone method with a calcium kit.³³⁸

**Statistical Analyses**

All experiments were done in triplicate. Significance was determined through one-way analysis of variance and Tukey test to evaluate multiple comparisons. Student t test was used to evaluate significance when only two conditions were analyzed, and significance was set at P < 0.05.

**RESULTS**

**HPLSC Characterization**

According to criteria of the International Society for Cellular Therapy,²⁸ human cells obtained from the middle third of the root were plastic adherent and expressed the positive mesenchymal stem cell markers STRO-1 and CD146 as evaluated by immunofluorescence (Fig. 1A). Furthermore, >95% of the cell population expressed mesenchymal stromal cell markers CD105, CD73, and CD90 as measured by flow cytometry (Figs. 1B and 1C). In addition, these cells lacked the expression of CD45, CD34, CD14, CD11b, and CD79 (2% to 3%) (Figs. 1B and 1C). Cell cultures were able to differentiate into adipose, cartilage, and bone lineages on treatment with specific differentiation media. After 21 days of incubation with chondrogenic media, cultures revealed clusters of adipose, cartilage, and bone lineages on treatment with specific differentiation media. After 21 days of incubation with adipogenic media after 21 days of incubation, as evidenced by oil red O stain (Fig. 1F). Thus, it can be concluded that the cell cultures were enriched in HPLSCs.

**PPP Has a Protein Profile Similar to That of PRP**

The current authors first aimed to determine the relative expression of 44 proteins in both PPP and PRP plasma fractions by using a protein profiler array. Surprisingly, a similar pattern of growth factors was observed in both plasma fractions using this proteomic tool (Figs. 2A and 2B). High expression of growth factors was detected in cell proliferation or differentiation of mesenchymal cells.⁷,³⁴ The authors estimated an increase of almost two-fold in PPP and three times in PRP of PDGF-AA, PDGF-BB, PDGF-AB, insulin-like growth factor binding protein (IGFBP)-2 relative to internal control expression in this array (Figs. 2C and 2D). Also noted was a three-fold increase of EGF in PRP and only one-fold in PPP. In addition, the authors identified an increase in both fractions of one-fold in proteins involved in periodontal tissue repair such as IGFBP-1, vascular endothelial growth factor receptor (VEGFR)-2, and EGF receptor (EGF-R) (Figs. 2C and 2D). Finally, less than one-fold expression was observed in both fractions of other proteins such as heparin binding-epidermal growth factor (HB-EGF); hepatocyte growth factor (HGF); PDGF receptor (PDGFR)-α and PDGFR-β; insulin-like growth factor (IGF)-I and IGF-II; IGF binding protein IGFBP-3, and IGFBP-4; IGFR1 receptor (IGFR); vascular endothelial growth factor (VEGF); VEGF-D and its receptors (VEGFR-3); bone morphogenetic protein (BMP)-4 and BMP-7; placental growth factor (PIGF); transforming growth factor (TGF)-α and three isoforms (TGF-β1, -β2, and -β3); granulocyte colony-stimulating factor (G-CSF); granulocyte-macrophage CSF (GM-CSF); M-CSF; M-CSF receptor (M-CSFR); stem cell factor (SCF); SCF receptor (SCFR); amphiregulin (AR); fibroblast growth factor (FGF)-2, FGF-4, FGF-6 and FGF-7; basic nerve growth factor (bNGF); neurotrophin (NT)-3 and NT-4; and glial cell–derived neurotropic factor (GDNF) (Figs. 2C and 2D). These results allow the authors to suggest for the first time that PPP has a protein expression pattern very similar to that of PRP, including biomolecules involved in wound healing and tissue repair.³⁴–³⁶

To further quantify differences between growth factors in PRP and PPP, the protein levels of three PDGF isoforms and EGF and FGF-2 were analyzed by ELISA. Statistically significant differences were observed between PPP and PRP in all PDGF isoforms and EGF levels. A 10-fold increase in PDGF isoforms in PRP fractions was found compared to PPP (PDGF-AA [pg/mL]: 21,266 versus 2,419; PDGF-AB: 28,834 versus 2,713; PDGF-BB: 20,245 versus 3,425) (Figs. 3A through 3C). At the same time, an increase of almost 100 times of PDGF-AAB: 20,245 versus 3,425) (Figs. 3A through 3C). At the same time, an increase of almost 100 times of EFG amounts in PRP fractions was observed compared

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††† ab100624, ab100622, ab100623, ab99979, and ab100504, AbCam.

‡‡‡ American Type Culture Collection, Manassas VA.

§§§ BioVision, San Francisco, CA.
to PPP (11,696 versus 1,037 pg/mL) (Fig. 3D). However, FGF-2 levels were similar in both fractions (1,141 versus 885 pg/mL) (Fig. 3E). These results suggest that PPP contains a protein profile very similar to that of PRP, but with lower amounts of PDGFs and EGF.

PPP or PRP Induces a Similar Effect on Bone Differentiation in HPLSCs

To evaluate the effect of PPP on bone differentiation, HPLSCs were incubated for 14 or 21 days in the presence of osteogenic media and PPP, PRP, or FBS (Figs. 4A through 4D). At the end of treatments, calcium deposits were quantified. At 14 days of incubation, statistical differences were found between PPP 2.5% and PRP 2.5%, showing higher calcium deposits for PRP-treated cells. Also, treatments with PRP 5% or 10% induced higher calcium deposits compared to PPP 2.5% (Fig. 4B). Cells incubated with FBS (2.5%, 5%, or 10%) and osteogenic media also showed calcium deposits (Fig. 4A); however, a lower response was observed compared to PPP or PRP. Furthermore, statistical differences were found between FBS 2.5% and 5% or 10% (Fig. 4A). At 21 days of HPLSC bone/cementum differentiation process, calcium deposits were found in all
treatments. Statistical differences were found between PPP 2.5% versus PRP 10% and PPP 5% versus PRP 10%, showing more calcium deposits in the cells incubated with PPP (Fig. 4D). No statistical differences were observed among all the other treatments. These observations suggest an evident effect of PPP on bone differentiation of HPLSCs, even at low concentrations of this platelet fraction.

Also analyzed were the calcium deposits in all HPLSC monolayers after 21 days of differentiation with alizarin red stain, confirming the presence of calcium deposits after PPP incubation (Figs. 4E and 4F). As a control, bone differentiation was induced in the human osteosarcoma cell line Saos-2, showing that PPP stimulated the formation of nodules with alizarin red staining (Fig. 4G). These results suggest a positive effect of PPP on bone/cementum differentiation of HPLSCs, inducing calcium deposits even at low PPP concentrations (2.5%).

**DISCUSSION**

This study reports the positive effect of PPP and PRP on bone differentiation using HPLSCs. The authors were able to obtain and characterize HPLSCs, satisfying the criteria defined by the International Society for Cellular Therapy to establish mesenchymal stem cells. The identification of appropriate biomolecules that control migration, proliferation, or differentiation of periodontal
ligament stem cells is a very important step in periodontal regeneration. In the periodontal regeneration field, platelet concentrates have been used as a source of growth factors to induce cellular responses in a disturbed environment.

It is important to consider that two systematic reviews that included 22 randomized clinical trials have recently reported contradictory results concerning the potential regenerative effect of PRP on periodontal wound healing. Therefore, it is important to understand the biologic rationale underlying the potential effect of these platelet-derived fractions. Although PRP contains increased amounts of growth factors compared to PPP, these lower quantities were enough to induce bone/cementum differentiation in vitro. In the present study, surprisingly, the authors found a similar protein profile for both PRP and PPP plasma fractions, using a proteome profiler antibody array with 44 proteins. PRP and PPP contain proteins related to migration, proliferation, remodeling, and differentiation in several tissues. To date, there are few reports related to biomolecules on PPP; therefore the present results are remarkable because they show that platelet-derived fractions could be used as an autologous source of different biomolecules. However, the results of quantitative ELISA analyses are similar to other reports. Interestingly, a recent study from Hatakeyama et al. showed the use of PPP as an effective biomaterial for the preservation of sockets with buccal dehiscence in dogs. In this study, PPP demonstrates the best results in terms of bone volume and width retention at the alveolar crest compared to PRP or platelet-rich fibrin. In addition, a clinical study performed by Yilmaz et al. reported similar clinical and radiographic bone gain in the treatment of intraosseous defects comparing PRP and PPP. Moreover, a recent study reported that PPP and PRP may similarly stimulate cell migration, myofibroblast differentiation, collagen remodeling, and type I collagen production in human gingival fibroblasts.

A striking result from the present study is the difference in the magnitude of growth factor levels determined with the proteomic antibody array and the ELISA assays. This difference may be due to distinct strategies used to quantitate in the array system (quantifying fold changes relative to the positive control) and ELISA (using single proteins as calibration curves with known values).

Scientific evidence from literature reports and the present results suggest that both PPP and PRP may exert a similar effect on periodontal wound healing events. These results may contradict the rationale of obtaining high concentrations of growth factors to obtain the best results in terms of tissue regeneration. Further studies are needed to understand the appropriate concentrations of

Figure 3. Growth factor quantification in PPP or PRP. Representative growth factors were quantified by ELISA as indicated. A) PDGF-BB, B) PDGF-AA, C) PDGF-AB, D) EFG, and E) FGF-2. Statistical differences were determined by Student t test: * P < 0.0007.
PPP induces bone differentiation even at low concentrations. HPLSCs were incubated for 14 or 21 days in the presence of osteogenic factors and different concentrations of PPP or PRP (2.5%, 5%, 10%). At the end of the treatments, calcium deposits were quantified. As a control, incubations with FBS were used with or without osteogenic factors. Calcium quantification in controls with FBS shows deposits in medium supplemented with osteogenic factors at 14 (A) or 21 (B) days of treatment. C Calcium deposits at 14 days with PPP or PRP treatment; statistical differences were observed (PPP 2.5% versus PRP 2.5% or PRP 5% or PPP 10%). D At 21 days of treatment, PPP induced higher calcium deposits; statistical differences were observed (PPP 2.5% versus PRP 10% and PPP 5% versus PRP 10%). Calcium deposits were visualized with alizarin red stain. Representative images of cell culture plates of HPLSCs treated with FBS (E) or PPP (F) at different concentrations. Also analyzed was the effect of PPP on osteosarcoma cell line Saos-2 as a control (G). Statistical differences were analyzed with one-way analysis of variance and Tukey test: * P < 0.05, † P < 0.0005, ‡ P < 0.0001. Values are shown as mean ± SEM of four independent experiments. O = osteogenic media.
growth factors necessary to achieve the best biologic and clinical responses.

CONCLUSIONS
These results suggest that PPP and PRP contain a similar profile of growth factors. Although PRP showed increased concentrations of these bioactive molecules, in vitro experiments showed a similar response for PPP and PRP on bone/cementum differentiation. To the authors’ knowledge, this is the first report that suggests that PRP and PPP may exert a similar response on bone differentiation of HPLSCs. Further studies in preclinical models and well-designed clinical trials are needed to characterize and compare the in vivo effect of PPP and PRP on bone healing.

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