Optimized Platelet Rich Fibrin With the Low Speed Concept: Growth Factor Release, Biocompatibility and Cellular Response

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Background: Over the past decade, the use of leukocyte platelet rich fibrin (L-PRF) has gained tremendous momentum in regenerative dentistry as a low-cost fibrin matrix utilized for tissue regeneration. In this study, we characterized how centrifugation speed (G-force) along with centrifugation time influence growth factor release from fibrin clots, as well as the cellular activity of gingival fibroblasts exposed to each PRF matrix.

Methods: Standard L-PRF served as a control (2700rpm-12 minutes). Two test groups utilizing low-speed (1300rpm-14 min termed advanced-PRF, A-PRF) and low-speed+time (1300rpm-8 min; A-PRF+) were investigated. Each PRF matrix was tested for growth factor release up to 10 days (8 donor samples) as well as biocompatibility and cellular activity.

Results: The low speed concept (A-PRF, A-PRF+) demonstrated a significant increase in growth factor release of PDGF, TGF- β1, EGF and IGF with A-PRF+ being highest of all groups. While all PRF formulations were extremely biocompatible due to their autogenous sources, both A-PRF and A-PRF+ demonstrated significantly higher levels of human fibroblast migration and proliferation when compared to L-PRF. Furthermore, gingival fibroblasts cultured with A-PRF+ demonstrated significantly higher mRNA levels of PDGF, TGF-β and collagen1 at either 3 or 7 days.

Conclusions: The findings from the present study demonstrate that modifications to centrifugation speed and time with the low-speed concept was shown to favor an increase in growth factor release from PRF clots which in turn may directly influence tissue regeneration by increasing fibroblast migration, proliferation and collagen mRNA levels. Future animal and clinical studies are now necessary.

KEYWORDS:

Fibrin, Blood, platelets, Regeneration, Wound healing, fibroblasts.

Over 15 years ago, platelet rich fibrin (PRF) was introduced as an autogenous source of blood growth factors that could serve as a tool for tissue regeneration in modern medicine.[1] The concepts were derived from the fact that a first generation platelet concentrate, termed platelet rich plasma (PRP), was being heavy utilized in various fields

of medicine despite bearing the negative aspect of containing anti-coagulants, thereby preventing the full coagulation cascade important for tissue wound healing.[2-4] PRF (which has since been renamed leukocyte-PRF or L-PRF due to its higher leukocyte content) does not contain any anti-coagulants and further provides a three-dimensional fibrin matrix that may be utilized as a scaffold for a variety of procedures including serving the function as a barrier membrane in guided bone and tissue regeneration (GBR, GTR) procedures.[5-7]

Since its introduction in 2001,[1] PRF has been extensively utilized in dentistry for a variety of procedures demonstrating its effectiveness for extraction socket management,[8] gingival recessions,[9-11] intrabony defect regeneration[12, 13] and sinus elevation procedures.[7] Major advantages include having completely immune-compatible growth factors collected at relatively no costs without anti-coagulants.[14-17] While initial and early experiments revealed that PRP contained high concentrations of autologous growth factors (up to 6-8 times higher than normal blood concentrations) including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and transforming growth factor-beta1 (TGF-β1),[18] PRF has since been shown to release even higher total growth factors over a more extended period of time.[19]

One of the primary proposed reasons for a slower release of growth factors over time, is the ability for the fibrin matrix to hold proteins within its fibrin network as well contains cells capable of further release growth factors into their surrounding micro-environment.[20-24] Leukocytes have been shown to be highly important immune cells capable of directing and recruiting various cell types during the wound healing process.[25-27] Since high centrifugation forces are known to shift cell populations to the bottom of collection tubes (whereas PRF is collected from the top one-third layer), it was recently hypothesized that by reducing centrifugation G-force, an increase in leukocyte numbers may be achieved within the PRF matrix.[28] It was since shown that by decreasing centrifugation g-force (now termed advanced-PRF or A-PRF) an increase in total leukocyte numbers within PRF matrix scaffolds was observed.[28] Furthermore and in agreement with this hypothesis, it was further shown that the release of several growth factors including PDGF, TGF-β1, VEGF, as well as epidermal growth factor (EGF), and insulin-like growth factor (IGF) were significantly higher in A-PRF when compared to L-PRF and PRP.[19]

Since centrifugation force has been shown to have a direct impact on growth factor release from within PRF scaffolds,[19] the aim of the present study was to further investigate whether centrifugation time would similarly further improve growth factor release from within PRF scaffolds. In principle, less centrifugation time would reduce cell pull-down by centrifugation forces, which would theoretically increase the total number of cells left contained within the top layer (PRF matrix). Furthermore, since at present it remains completely unknown what influence these changes to centrifugation protocols will have on tissue regeneration, the effects of each PRF matrix including L-PRF, A-PRF and A-PRF+ was investigated for the first time on human gingival fibroblast cell biocompatibility and cell activity. Cells were therefore cultured with growth factors from each PRF matrix (L-PRF, A-PRF and A-PRF+) and investigated for cell migration, proliferation, growth factor release and collagen synthesis *in vitro.*

MATERIALS AND METHODS

The Institutional Review Board (IRB) of Nova Southeastern University (Fort Lauderdale, Florida) determined that this study did not require IRB review or approval.

Platelet Concentrations

Blood samples were collected with the informed consent of 8 volunteer donors (24 total samples) and blood was then processed for L-PRF, A-PRF, A-PRF+ centrifugation. All blood samples were obtained from members of our laboratory between the ages of 30 and 60. Ten ml of whole blood without anticoagulant was centrifuged at 2,700 rpm (708g) for 12 min for L-PRF, at 1,300 rpm (200g) for 14 min for A-PRF, and at 1,300 rpm (200g) for 8 min for A-PRF+ respectively by a centrifuge machine[#].[19, 28] The size and volume of the L-PRF, A-PRF and A-PRF+ clots were produced in the top 4ml layer of the centrifuge tubes (4ml out of 10ml). The PRF clot was then removed and placed into a 6-well dish accordingly with 5 ml of DMEM culture media[**] and processed as further described according to our previous study.[19]

Protein Quantification With ELISA

In order to determine the amount of released growth factors from L-PRF, A-PRF, and A-PRF+ at 15 min, 60 min, 8 hours, 1 day, 3 days and 10 days, samples were placed into a shaking incubator at 37°C to allow for growth factor release into the culture media. At each time point, the 5 ml of culture media was collected, frozen and replaced with 5 ml of additional culture media. Protein quantification was carried out using ELISA. At desired time points, PDGF-AA (DY221, range = 15.60 - 1,000 pg/ml), PDGF-AB (DY222, range = 15.60 - 1,000 pg/ml), PDGF-BB (DY220, 31.20 - 2,000 pg/ml), TGF-β1 (DY240, range = 31.20 - 2,000 pg/ml), VEGF (DY293B, range = 31.20 - 2,000 pg/ml), EGF (DY236, range = 3.91 - 250 pg/ml) and IGF-1 (DY291, range = 31.20 - 2,000 pg/ml) were quantified using an ELISA kit[††] according to manufacturer's protocol as previously described.[19] Absorbance was measured at 450 nm and 570 nm using a microplate reader[‡‡] and subtract at 570 nm from the readings at 450 nm. All samples were measured in duplicate and 8 independent experiments were performed for each platelet concentrate.

Cell Culture

Platelet concentrates including L-PRF, A-PRF, and A-PRF+ were incubated for 3 days on a plate shaker at 37 °C as previously described[19] and thereafter conditioned media was collected and utilized in future experiments as 20% of the total volume. All cell culture experiments were cultured with 20% CM in standard DMEM cell culture media containing 15% FBS. Human gingival fibroblasts[§§] were cultured in a humidified atmosphere at 37°C in growth medium consisting of DMEM[**], 10% fetal bovine serum (FBS)[**], and 1% antibiotics[**] and used for experimental seeding from passages 4-6. All cells were detached from tissue culture plastic using 0.25% EDTA-Trypsin[**] prior to reaching confluency. Cells were seeded with 20% conditioned media from L-PRF, A-PRF and A-PRF+ in contained within growth medium at a density of 10,000 cells for cell viability and proliferation experiments and 50,000 cells per well for real-time PCR experiments in 24-well plates. Control samples are cells seeded onto tissue culture plastic alone that contained 20% conditioned media left for 3 days on a plate shaker at 37 °C

without PRF clots (completely blank samples). For experiments lasting longer than 5 days, medium was replaced twice weekly.

Cell Viability

At 24 hours post cell seeding, cells were evaluated using a live-dead staining assay[‖‖] according to the manufacturer's protocol. Fluorescent images were quantified with an inverted fluorescent microscope[¶¶]. Thereafter, cells were expressed as percentages of live versus dead cells following cell culture growth with L-PRF, A-PRF and A-PRF+.

Cell Migration Assay

The migration assay of human gingival fibroblasts was performed using 24-well plates and polyethylene terephthalate cell culture inserts with a pore size of 8 μm[##]. The platelet conditioned media were filled into the lower compartment of the wells. After starved in DMEM containing 0.5% FBS for 12 hours, 10,000 cells were seeded in the upper compartment. After 24 hours, cells were fixed with 4% formaldehyde for 2 min. Thereafter cells were permeabilized by acetone for 15 min and stained with hematoxylin solution[***] for 20 min. The upper side of the filter membrane were rinsed and gently wiped by a cotton swab to remove the cell debris. The numbers of cells on the lower side of the filter were counted under a microscope[¶¶].

Proliferation Assay

Human gingival fibroblasts were quantified using a MTS colorimetric assay[†††] at 1, 3 and 5 days for cell proliferation as previously described.[29] At desired time points, cells were washed with phosphate buffered solution (PBS) and quantified using a microplate reader[‡‡].

Real-Time PCR Analysis

Total RNA was harvested at 3 and 7 days post stimulating for human gingival fibrobalsts to investigate mRNA levels of TGF-β, PDGF and collagen1a2 (COL1a2). Primer and probe sequences for genes were fabricated with primer sequences according to Table 1. RNA isolation was performed using High Pure RNA Isolation Kit[‡‡‡]. Real-time RT-PCR was performed using Roche Master mix[‡‡‡] and quantified on the StepOne™ plus Real time PCR system[§§§]. The ΔΔCt method was used to calculate gene expression levels normalized to the expression of GAPDH.

Statistical Analysis

All experiments were performed in triplicate with three independent experiments for each condition. Means and standard errors (SE) were calculated and data were analyzed for statistical significance using one-way analysis for cell viability and migration assay, two-way analysis of variance for ELISA, proliferation assay and real time PCR analysis with Turkey test (p values < 0.05 was considered significant) by GraphPad Prism 6.0 software[‖‖‖].

RESULTS

Growth Factor Release From PRF, A-PRF and A-PRF+

In a first set of experiments, the release of growth factors including PDGF-AA, PDGF-AB, PDGF-BB, TGF-β1, VEGF, EGF and IGF-1 were quantified by ELISA (Fig. 1, 2). While the growth factor release of PDGF-AA demonstrated a significant increase for A-PRF+ at 3 days when compared to L-PRF (Fig. 1A), no difference in total growth factor released was observed between the 3 treatment groups (Fig. 1B). Release of PDGF-AB demonstrated a significant increase for A-PRF+ when compared to PRF at 8 hours, and a significant increase at 1, 3 and 10 days when compared to all other groups (Fig. 1C). L-PRF demonstrated significantly lower values than A-PRF and A-PRF+ at both 1 and 3 days (Fig. 1C). The total growth factor release was significantly higher for A-PRF+ when compared to all modalities while L-PRF was significantly lowest (Fig. 1D). Similarly, the release of PDGF-BB demonstrated significantly highest values at almost all time points for A-PRF+, with L-PRF once again demonstrating significantly lower values when compared to A-PRF and A-PRF+ (Fig. 1E, 1F).

The release of TGF-β1 also demonstrated a similar trend whereby A-PRF+ demonstrated significantly highest values at 1, 3 and 10 days (Fig. 2A) and the total release of growth factors after a 10-day period almost 3 times significantly higher when compared to L-PRF (Fig. 2B). Interestingly, A-PRF+ demonstrated a significantly higher release of VEGF at an early time point of 1 day (Fig. 2C) whereas little change was observed in the total growth factor release (Fig. 2D). Release of growth factors EGF and IGF-1 confirmed that the low speed concept favoured the release of both growth factors from A-PRF+ when compared to A-PRF and L-PRF (Fig. 2E-H).

Biocompatibility of L-PRF, A-PRF and A-PRF+ on Human Gingival Fibroblasts

In a first cell culture experiment the influence of L-PRF, A-PRF and A-PRF+ was investigated on cell viability of human gingival fibroblasts. It was found that all platelet formulations displayed excellent cell biocompatibility by demonstrating most notably high living cells (green cells, Fig, 3) with very few observable apoptotic cells (red cells). It may therefore be concluded from this experiment that each platelet rich fibrin formulation including L-PRF, A-PRF and A-PRF+ are fully biocompatible under the present in vitro cell culture model (Fig. 3).

Influence of PRF, A-PRF and A-PRF+ on Human Gingival Fibroblast Activity

Following growth factor release experiments, each PRF formulation was investigated on gingival fibroblast cell migration, proliferation, mRNA expression of growth factors and collagen (Figs. 4-5). It was first observed that all PRF matrix scaffolds were able to significantly promote gingival fibroblast migration at 24 hours with L-PRF inducing a 200% increase whereas A-PRF and A-PRF+ induced a 300% increase (Fig. 4A). No significant difference between A-PRF and A-PRF+ was observed although both were significantly higher than control tissue culture plastic and L-PRF (Fig. 4A). A similar trend was also observed for cell proliferation where each of L-PRF, A-PRF and A-PRF+

significantly increase cell numbers at both 3 and 5 days when compared to control tissue culture plastic, and A-PRF and A-PRF+ were significantly higher than all other groups at 5 days only (Fig. 4B).

Thereafter, mRNA levels of common regenerative cytokines PDGF and TGF-β were evaluated by real-time PCR (Fig. 5). It was first found that A-PRF and A-PRF+ provoked a slight significant increase in PDGF mRNA levels at 3 days when compared to control tissue culture plastic (Fig. 5A). At 7 days, L-PRF demonstrated a significant increase when compared to control tissue culture plastic whereas both A-PRF and A-PRF+ once again provoked a significant increase in PDGF mRNA levels when compared to all other groups (Fig. 5A). No significant difference was observed between A-PRF and A-PRF+ at either time point (Fig. 5A). Thereafter growth factor release of TGF-β from gingival fibroblasts was investigated (Fig. 5B). It was observed that while no difference was present at 3 days post-seeding, a significant increase was observed for all PRF groups at 7 days (Fig. 5B). It was found that A-PRF+ demonstrated significantly highest mRNA levels at 7 days when compared to all other groups including control tissue culture plastic, L-PRF as well as A-PRF (Fig. 5B).

Lastly, collagen mRNA levels were quantified by real-time PCR (Fig. 5C). At 3 days, mRNA levels of collagen1 were significantly higher in A-PRF and A-PRF+ groups when compared to control tissue culture plastic with no significant differences observed for L-PRF (Fig. 5C). At 7 days post seeding, L-PRF demonstrated significantly higher values when compared to control tissue culture plastic and A-PRF demonstrated statistically significantly higher values when compared to control tissue culture plastic and L-PRF (Fig. 5C). A-PRF+ demonstrated significantly highest mRNA values when compared to all other groups (Fig. 5C).

DISCUSSION

The aim of the present study was to investigate the influence of centrifugation speed (g-force) and time on PRF matrix scaffolds, their release of growth factors as well as their effect on cellular biocompatibility and activity. As the use of PRF has continuously and steadily increased in regenerative implant dentistry and periodontology, there remains great clinical benefit to optimize centrifugation protocols for clinical practice. Therefore, the aim of the present study was to investigate if lower centrifugation speeds and time could be additionally used to improve growth factor release and cell bioactivity. One of the interesting findings from a previous study by Ghanaati et al. found that cells quantified histologically within the PRF matrix observed that the majority of leukocytes were found near the bottom of the fibrin clot in standard L-PRF.[28] Based on this finding, it became clear that centrifugation speeds (G-forces) were evidently too high pushing leukocytes down to the bottom of centrifugation tubes and away from the PRF matrix clot. In order to redistribute leukocyte cell numbers across the entire PRF matrix, lower centrifugation speeds were investigated.[28] It was confirmed that higher cell number could be obtained by reducing G-force during centrifugation.[28] Ghanaati et al. showed that while platelets were detected throughout the clot in both groups (L-PRF and A-PRF), more platelets were found in the distal part of A-PRF.[28] Furthermore, by decreasing the rpm while increasing the centrifugation time in the A-PRF group, an enhanced presence of neutrophilic granulocytes in the distal part of the clot was also observed.[28]

Accordingly, it was reported that a higher presence of these cells might be able to influence the differentiation of host macrophages and macrophages within the clot after implantation.[28] Thus, it was concluded that A-PRF might influence bone and soft tissue regeneration, especially through the presence of monocytes/macrophages and their growth factors.[28] In theory, the practical application of these new centrifugation protocols are derived by minimizing centrifugation speeds in order to limit the centrifugation pull-down of leukocyte cells to the lower compartment of centrifugation tubes. By reducing centrifugation g-force and time, a higher percentage of cells can therefore be collected within the top layer where PRF clots are located and utilized clinically. It remains to be investigated in a future study the histological features and cell numbers of A-PRF+ in comparison to L-PRF and A-PRF.

It must also be noted that the role of leukocytes in tissue wound healing and bone biology has been extensively discussed and critically important to wound healing.[25-27] Interesting findings from basic science now point to the absolute necessity of macrophages during bone tissue remodelling[30] and have further shown that macrophages are responsible for a 23-fold increase in osteoblast differentiation.[31] Without these key immune cells, it has been shown that bone formation has very limited potential to generate new bone.[30] Furthermore, macrophages are key players in biomaterial integration and are the responsible cell type dictating material integration.[32] Therefore it becomes evident that both an increase in leukocyte number as well as their even distribution across the PRF scaffold as demonstrated with lower centrifugation speeds is highly favourable during tissue wound healing and during biomaterial integration of collagen barrier membranes, various classes of bone grafting materials and potentially dental implants.[32] Future research is therefore necessary.

Another important aspect of leukocyte biology that has not been discussed in this study but again shows much clinical relevance is the fact leukocytes are the responsible cell-type acting to prevent infiltrating pathogens.[33, 34] In light of this fact, it becomes of interest to note that PRF placed into extraction sockets has been shown to greatly decrease the rate of complications and infections.[8] Hoaling and Lines reported that filling 3rd molar extraction sockets with PRF led to a 10 fold decrease in osteomyelitis infections when compared to natural healing.[8] This study performed in 200 patients utilized bilateral extractions (one side filled with PRF, the other left to naturally heal) providing good scientific evidence for the reduced rate of infection following healing with PRF.[8]

In the present study, we first investigated growth factor release from the various PRF matrix scaffolds produced by 3 different centrifugation protocols utilizing a slower-speed concept (Fig. 1, 2). It was reported that A-PRF+ demonstrated significantly higher total growth factor release when compared to both A-PRF as well as L-PRF. We therefore hypothesized that this finding is directly correlated with the fact that a higher number of leukocytes are found contained within the A-PRF+ scaffolds centrifuged utilizing lower G-forces and centrifugation times. This finding alone is deemed highly clinically relevant and these slight changes in centrifugation protocols were shown to have a direct and pronounced impact on growth factor release from within these A-PRF+ scaffolds. One aspect that remains to be investigated is how cytokine profiles of A-PRF and A-PRF+ would compare to L-PRF. Since the most commonly found growth factors and cytokines

in PRF as those investigated from previous work conducted over a decade ago from the original L-PRF formulation,[35] it remains of interest to our group to determine if not only higher concentrations of growth factors are released from the various PRF formulations, but also if additional growth factors or cytokines may also be subsequently released. Future research utilizing cytokine prolife assays comparing the various PRF formulations would be necessary to further investigate these possible differences.

Another interesting area of research that is often left unstudied is the effect of higher than optimal doses of growth factors on tissue remodelling. For instance, Oshima et al. found that certain growth factors including TGF-beta and VEGF are not only capable of supporting tissue regeneration but may also participate in tissue degradation in periodontitis.[36, 37] While in general both of these growth factors are routinely associated with tissue regrowth (TGF-beta) and angiogenesis (VEGF), it must also not be excluded that they may also show negative effects also. Future research investigating the optimal growth factor concentrations from PRF formulations remains to be determined.

Following the analysis of growth factor release from PRF matrix scaffolds, our group then sought to characterize the influence of L-PRF, A-PRF and A-PRF+ on cell biocompatibility and cell activity (Fig. 3-5). It was first found that all PRF centrifugation protocols led to extremely high biocompatibility due to the autogenous source of these growth factors without use of anti-coagulants (Fig. 3). Interestingly, it was found that A-PRF and A-PRF+ significantly promoted higher human gingival cell migration and proliferation when compared to control tissue culture plastic and L-PRF (Fig. 4). Furthermore, analysis of mRNA levels of PDGF and TGF-β also demonstrate the ability for PRF matrix scaffolds produced with the low speed concept to significantly increase the production of released growth factors from gingival fibroblasts. Therefore, not only are higher quantities of PDGF and TGF-β1 found in A-PRF+ scaffolds themselves (Fig. 1, 2), but the cells then in contact with their matrix are also further stimulated to release more growth factors (Fig. 5), thus having a synergistic effect on the total growth factors produced locally.

Lastly we showed also that A-PRF and A-PRF+ samples were able to locally increase in collagen1 mRNA levels (Fig. 5C). Not surprisingly, collagen remains one of the key factors during tissue wound healing and remodelling.[38] Therefore the increase in collagen type 1 when cells were exposed to A-PRF and A-PRF+ further demonstrates the regenerative potential of the newer PRF formulations centrifuged at lower g-forces and lower centrifugation times.

CONCLUSION

In summary, the results from the present study demonstrate that all formulations of PRF matrix scaffolds including PRF, A-PRF and A-PRF+ were able to secrete the local release of various growth factors important for tissue regeneration and A-PRF+ demonstrated significantly higher release of growth factors when compared to all other groups. Furthermore, A-PRF and A-PRF+ matrix scaffolds were shown to directly impact the ability for human gingival fibroblasts to migrate, proliferate, release additional growth factors and increase mRNA levels of type 1 collagen. The findings from the present study demonstrate that modifications to centrifugation speed and time with the low-speed concept favors an increase in growth factor concentrations directly impacting

human gingival fibroblasts. Future animal and clinical studies are now needed to further confirm the effects of these results in vivo.

REFERENCES

1. Choukroun J, Adda F, Schoeffler C, Vervelle A. Opportunities in Implant Dentistry: PRF (french). *Implantodontie* 2001;42:e62.
2. Anfossi G, Trovati M, Mularoni E, Massucco P, Calcamuggi G, Emanuelli G. Influence of propranolol on platelet aggregation and thromboxane B2 production from platelet-rich plasma and whole blood. *Prostaglandins, leukotrienes, and essential fatty acids* 1989;36:1-7.
3. Fijnheer R, Pietersz RN, de Korte D, et al. Platelet activation during preparation of platelet concentrates: a comparison of the platelet-rich plasma and the buffy coat methods. *Transfusion* 1990;30:634-638.
4. Marx RE. Platelet-rich plasma: evidence to support its use. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons* 2004;62:489-496.
5. Toffler M. Guided bone regeneration (GBR) using cortical bone pins in combination with leukocyte- and platelet-rich fibrin (L-PRF). *Compendium of continuing education in dentistry (Jamesburg, NJ : 1995)* 2014;35:192-198.
6. Lekovic V, Milinkovic I, Aleksic Z, et al. Platelet-rich fibrin and bovine porous bone mineral vs. platelet-rich fibrin in the treatment of intrabony periodontal defects. *Journal of periodontal research* 2012;47:409-417.
7. Shivashankar VY, Johns DA, Vidyanath S, Sam G. Combination of platelet rich fibrin, hydroxyapatite and PRF membrane in the management of large inflammatory periapical lesion. *Journal of Conservative Dentistry* 2013;16:261-4.
8. Hoaglin DR, Lines GK. Prevention of localized osteitis in mandibular third-molar sites using platelet-rich fibrin. *International journal of dentistry* 2013;2013:875380.
9. Aroca S, Keglevich T, Barbieri B, Gera I, Etienne D. Clinical evaluation of a modified coronally advanced flap alone or in combination with a platelet-rich fibrin membrane for the treatment of adjacent multiple gingival recessions: a 6-month study. *Journal of periodontology* 2009;80:244-252.
10. Padma R, Shilpa A, Kumar PA, Nagasri M, Kumar C, Sreedhar A. A split mouth randomized controlled study to evaluate the adjunctive effect of platelet-rich fibrin to coronally advanced flap in Miller's class-I and II recession defects. *Journal of Indian Society of Periodontology* 2013;17:631-636.
11. Agarwal SK, Jhingran R, Bains VK, Srivastava R, Madan R, Rizvi I. Patient-centered evaluation of microsurgical management of gingival recession using coronally advanced flap with platelet-rich fibrin or amnion membrane: A comparative analysis. *European journal of dentistry* 2016;10:121-133.
12. Agarwal A, Gupta ND, Jain A. Platelet rich fibrin combined with decalcified freeze-dried bone allograft for the treatment of human intrabony periodontal defects: a randomized split mouth clinical trail. *Acta odontologica Scandinavica* 2016;74:36-43.
13. Elgendy EA, Abo Shady TE. Clinical and radiographic evaluation of nanocrystalline hydroxyapatite with or without platelet-rich fibrin membrane in the treatment of periodontal intrabony defects. *Journal of Indian Society of Periodontology* 2015;19:61-65.
14. Medina-Porqueres I, Alvarez-Juarez P. The Efficacy of Platelet-Rich Plasma Injection in the Management of Hip Osteoarthritis: A Systematic Review Protocol. *Musculoskeletal care* 2016 Jun;14(2):121-5.
15. Salamanna F, Veronesi F, Maglio M, Della Bella E. New and emerging strategies in platelet-rich plasma application in musculoskeletal regenerative procedures: general overview on still open questions and outlook. BioMed Research International. 2015;2015:846045.

16. Albanese A, Licata ME, Polizzi B, Campisi G. Platelet-rich plasma (PRP) in dental and oral surgery: from the wound healing to bone regeneration. *Immunity & ageing : I & A* 2013;10:23.

17. Panda S, Doraiswamy J, Malaiappan S, Varghese SS, Del Fabbro M. Additive effect of autologous platelet concentrates in treatment of intrabony defects: a systematic review and meta-analysis. *Journal of investigative and clinical dentistry* 2014.

18. Peerbooms JC, van Laar W, Faber F, Schuller HM, van der Hoeven H, Gosens T. Use of platelet rich plasma to treat plantar fasciitis: design of a multi centre randomized controlled trial. *BMC Musculoskeletal Disorders* 2010;11:69-69.

19. Kobayashi E, Fluckiger L, Fujioka-Kobayashi M, et al. Comparative release of growth factors from PRP, PRF, and advanced-PRF. *Clinical oral investigations* 2016.Jan 25. [Epub ahead of print]

20. Lekovic V, Milinkovic I, Aleksic Z, et al. Platelet-rich fibrin and bovine porous bone mineral vs. platelet-rich fibrin in the treatment of intrabony periodontal defects. *Journal of periodontal research* 2012;47:409-417.

21. Panda S, Jayakumar ND, Sankari M, Varghese SS, Kumar DS. Platelet rich fibrin and xenograft in treatment of intrabony defect. *Contemporary clinical dentistry* 2014;5:550-554.

22. Pradeep AR, Rao NS, Agarwal E, Bajaj P, Kumari M, Naik SB. Comparative evaluation of autologous platelet-rich fibrin and platelet-rich plasma in the treatment of 3-wall intrabony defects in chronic periodontitis: a randomized controlled clinical trial. *Journal of periodontology* 2012;83:1499-1507.

23. Sharma A, Pradeep AR. Treatment of 3-wall intrabony defects in patients with chronic periodontitis with autologous platelet-rich fibrin: a randomized controlled clinical trial. *Journal of periodontology* 2011;82:1705-1712.

24. Kumar RV, Shubhashini N. Platelet rich fibrin: a new paradigm in periodontal regeneration. *Cell and tissue banking* 2013;14:453-463.

25. Bielecki T, Dohan Ehrenfest DM, Everts PA, Wiczkowski A. The role of leukocytes from L-PRP/L-PRF in wound healing and immune defense: new perspectives. *Current pharmaceutical biotechnology* 2012;13:1153-1162.

26. Martin P. Wound healing--aiming for perfect skin regeneration. *Science* 1997;276:75-81.

27. Barrick B, Campbell EJ, Owen CA. Leukocyte proteinases in wound healing: roles in physiologic and pathologic processes. *Wound Repair and Regeneration* 1999;7:410-422.

28. Ghanaati S, Booms P, Orlowska A, et al. Advanced platelet-rich fibrin: a new concept for cell-based tissue engineering by means of inflammatory cells. *The Journal of oral implantology* 2014;40:679-689.

29. Fujioka-Kobayashi M, Sawada K, Kobayashi E, Schaller B, Zhang Y, Miron RJ. Osteogenic potential of rhBMP9 combined with a bovine-derived natural bone mineral scaffold compared to rhBMP2. *Clinical oral implants research* 2016.

30. Sinder BP, Pettit AR, McCauley LK. Macrophages: Their Emerging Roles in Bone. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 2015;30:2140-2149.

31. Chang MK, Raggatt LJ, Alexander KA, et al. Osteal tissue macrophages are intercalated throughout human and mouse bone lining tissues and regulate osteoblast function in vitro and in vivo. *Journal of immunology (Baltimore, Md : 1950)* 2008;181:1232-1244.

32. Miron RJ, Bosshardt DD. OsteoMacs: Key players around bone biomaterials. *Biomaterials* 2016;82:1-19.

33. Ramilo O, Allman W, Chung W, et al. Gene expression patterns in blood leukocytes discriminate patients with acute infections. *Blood* 2007;109:2066-2077.

34. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nature reviews immunology* 2008;8:958-969.

35. Dohan DM, Choukroun J, Diss A, et al. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part II: platelet-related biologic features. *Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics* 2006;101:e45-50.

36. Ohshima M, Yamaguchi Y, Matsumoto N, et al. TGF-beta signaling in gingival fibroblast-epithelial interaction. *Journal of dental research* 2010;89:1315-1321.

37. Ohshima M, Yamaguchi Y, Ambe K, et al. Fibroblast VEGF-receptor 1 expression as molecular target in periodontitis. *Journal of clinical periodontology* 2016;43:128-137.

38. Chattopadhyay S, Raines RT. Review collagen-based biomaterials for wound healing. *Biopolymers* 2014;101:821-833.

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Figure 1:

ELISA protein quantification at each time point of (A) PDGF-AA, (C) PDGF-AB and (E) PDGF-BB over a 10 day period. Total accumulated growth factor released over a 10 day period for (B) PDGF-AA, (D) PDGF-AB and (F) PDGF-BB. ($p<0.05$ signifies significant difference between groups, ** $p<0.05$ signifies significantly higher than all other groups, # $p<0.05$ signifies significantly lower than all groups).*

Figure 2:

ELISA protein quantification at each time point of (A) TGF-β1, (C) VEGF (E) EGF and (G) IGF-1 over a 10 day period. Total accumulated growth factor released over a 10 day period for (B) TGF-β1, (D) VEGF, (F) EGF and (H) IGF-1. ($p<0.05$ signifies significant difference between groups, ** $p<0.05$ signifies significantly higher than all other groups, # $p<0.05$ signifies significantly lower than all groups).*

Figure 3:

Live/Dead assay at 24 hours of human gingival fibroblasts treated with L-PRF, A-PRF or A-PRF+ (A) The merged fluorescent images of Live/Dead staining with viable cell appearing in green and dead cells in red. (B) Cell viability was quantified with percentage of numbers of living cells in each group. No significant changes in cell viability were observed for all platelet concentrates.

Figure 4:

Effects of L-PRF, A-PRF and A-PRF+ on human gingival fibroblast (A) cell migration at 24 hours and (B) cell proliferation at 1, 3 and 5 days. (denotes significantly higher than control group $p<0.05$, ** denotes significantly higher than all other groups $p<0.05$)*

Figure 5:

Real-time PCR of human gingival fibroblasts treated with L-PRF, A-PRF or A-PRF+ at 3 and 7 days for mRNA levels of (A) TGF-β, (B) PDGF and (C) COL1a2. (denotes significant difference between groups, $p<0.05$, ** denotes significantly higher than all other groups $p<0.05$)*

Table 1:

List of primer sequences for real-time PCR

Gene	Primer Sequence
hTGF-β F	actactacgccaaggaggtcac
hTGF-β R	tgcttgaacttgtcatagatttcg
hPDGF F	cacacctcctcgctgtagtattta
hPDGF R	gttatcggtgtaaatgtcatccaa

hCOL1a2 F	cccagccaagaactggtatagg
hCOL1a2 R	ggctgccagcattgatagtttc
hGAPDH F	agccacatcgctcagacac
hGAPDH R	gcccaatacgaccaaatcc

PRF Duo Centrifuge, Process for PRF, Nice, France

** Gibco, Life technologies, Carlsbad, CA, USA

†† DuoSet, R&D Systems, Minneapolis, MN, USA

‡‡ DTX880, Beckman Coulter, Brea, California, USA

§§ HGF-1, ATCC, Manassas, VA, USA

‖‖ Enzo Life Sciences AG; Lausen, Switzerland

¶¶ IX51, OLYMPUS, Tokyo, Japan

Falcon, Corning Inc., Corning, NY, USA

*** Sigma, St. Louis, MO, USA

††† Promega, Madison, WI, USA

‡‡‡ Roche, Basel, Switzerland

§§§ Applied Biosystems, Foster City, CA, USA

‖‖‖ GraphPad Software, Inc., La Jolla, CA, USA

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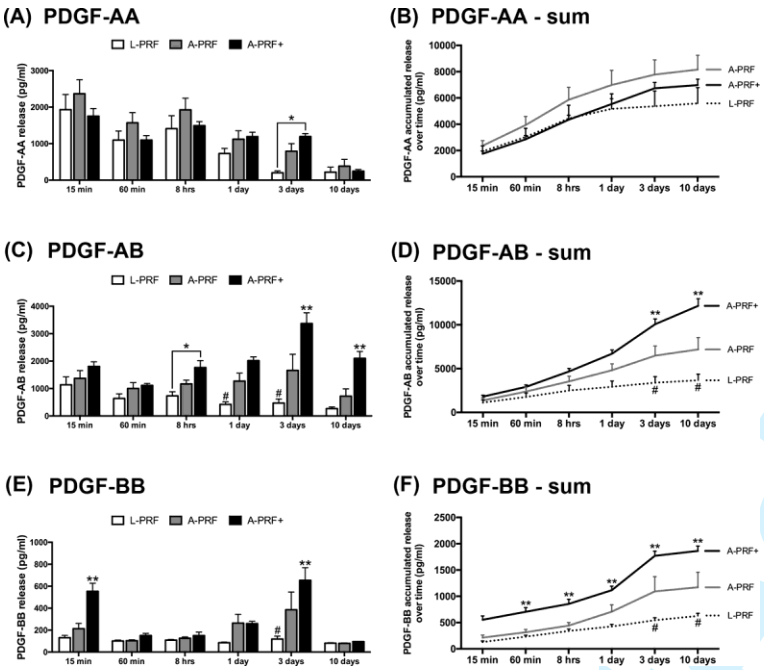
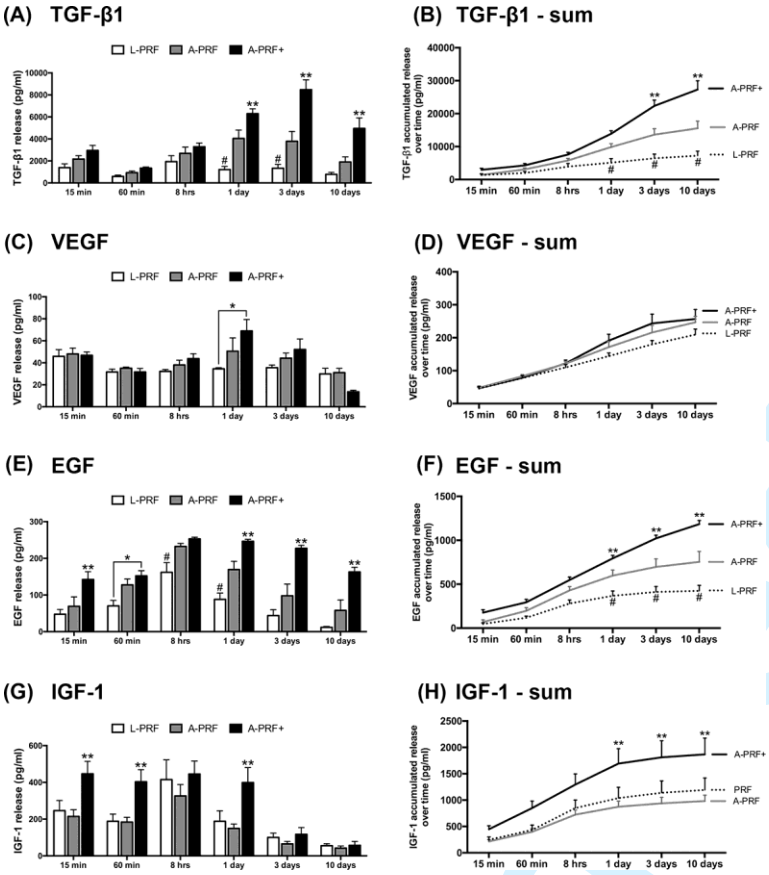
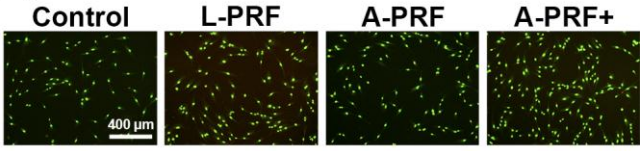
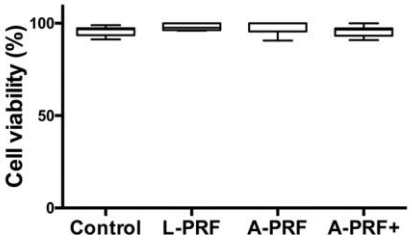




(A)

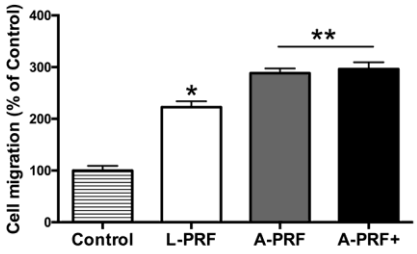

Control
L-PRF
A-PRF
A-PRF+
400 µm

(B)

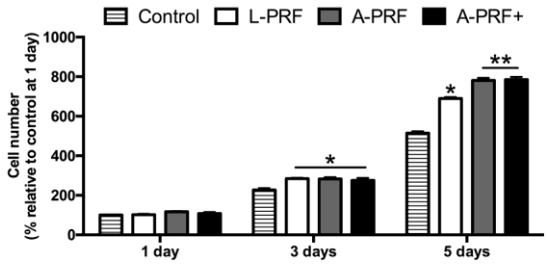

Cell viability (%)
100
50
0
Control
L-PRF
A-PRF
A-PRF+

 DOI: 10.1902/jop.2016.160443

(A)



(B)



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