

Platelet-rich Fibrin Influences on Proliferation and Migration of Human Gingival Fibroblasts

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ABSTRACT

Aims: Our study focused on the fabrication of platelet-rich fibrin (PRF) and evaluated its influences on cell behaviors, including proliferation and migration.

Materials and methods: Platelet-rich fibrin was prepared from human peripheral blood according to Choukroun's method without using nonanticoagulant and foreign factors for platelet activation. Platelet-rich fibrin architecture was studied by hematoxylin and eosin staining. The investigation of PRF effects on human gingival fibroblasts (hGFs) was conducted via PRF liquid extract. Cell proliferation was determined via the number of cells after a period of time incubated in PRF liquid extract. Influence of PRF liquid extract on the migration of hGFs was conducted via scratch wound healing assay.

Results: Histological staining reviewed the natural fibrin fiber matrix of PRF. Platelet-rich fibrin liquid extract promoted hGF proliferation after 7 days of cultivation. Human gingival fibroblast proliferation in PRF liquid extract was more superior than those cultured in complete medium. Platelet-rich fibrin was also found to be able to promote the migration of hGFs for up to 48 hours.

Conclusion: These results indicated that PRF is suitable to be used as autologous natural biomaterial in supporting wound healing and in further application in periodontitis treatments.

Keywords: Gingival fibroblast, Migration, Periodontitis treatment, Platelet-rich fibrin, Proliferation.

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INTRODUCTION

Periodontal inflammation is a common disease and is also a high risk factor for tooth loss all over the world.

Currently, the demand for periodontal treatment is quite high. A lot of treatments are conducted. However, the wound healing process takes a long time and the patients have to pay the high cost for treatment. Development of new materials to support the wound healing process in a short time and low cost used in the treatment of periodontal disease is an issue that needs to be studied. Healing process after surgery is an important factor to be considered to assess the effectiveness of clinical treatment. The activated elements combined as healing aids are signal proteins that regulate inflammatory responses and stimulate the wound healing process. Clot formation is the first phenomenon in the wound healing process. Platelets do not only have a role in preventing bleeding, but it is also one of the important factors stimulating wound healing. Ross et al,¹ first announced the regeneration ability of the platelets through the growth factors released from activated platelets in 1974. In 2005, Nevins et al² have used growth factors from platelets [platelet-derived growth factor (PDGF)] as a supporting factor for treating alveolar bone defect combined with tricalciumphosphate beta (β -TCP). The treatment combined with PDGF had restored alveolar bone defects after 3 months; half of the time was shortened than β -TCP therapy for 6 months. Thus, based on the capacity to release growth factors from activated platelets, their role helps in stimulating cell proliferation, new blood vessel formation, etc. The development of platelet-rich products attracted the attention of scientists for studying and applying platelets with high concentrations in wound healing. The first platelet-rich product is platelet-rich plasma (PRP). Platelet-rich plasma was created by Robert E Marx et al.³ Under this procedure, the blood was collected with anticoagulant and twice centrifuged to obtain plasma with high platelet concentration. The platelets in plasma were activated by adding bovine thrombin and calcium chloride to release growth factors. According to this study, the number of platelets in PRP was picked three times compared with normal platelet counted in the blood and PRP-enhanced bone regeneration in maxillofacial surgery. Many materials are used in combination with PRP in bone regeneration. However, the impact of PRP in stimulating bone healing in these studies still causes controversy.

The direct use of additional factors in collecting PRP can lead to the formation of adverse reactions or

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antibodies to coagulation factors, such as factors V, XI, and thrombin. Platelet-rich fibrin (PRF) is considered as the potential second-generation platelet-rich products.⁴ Platelet-rich fibrin was first published in France by Choukroun et al in 2001.⁵ Platelet-rich fibrin was applied more and more in order to support the wound healing process because of many advantages compared to the PRP, such as the simple and effective collecting process, no added substances from the outside to activate the platelets, arrested platelets into the fibrin network, and support for hemostasis, cell migration, and proliferation. Platelet-rich fibrin is obtained from the blood without anticoagulant and centrifuged just one-time to obtain PRF matrix. The platelets in the fibrin fiber network were activated to release growth factors without additional components, such as bovine thrombin and calcium chloride. Platelet-rich fibrin is applied in many clinical trials, but the mechanism of PRF to the wound healing has not been studied much. For developing applications of PRF in clinical treatment, the research about the scientific base of PRF should be promoted. The collection and application of PRF as a support material in the wound healing would be a new step for the treatment of periodontitis in the world, toward a safe, effective, less costly, and time-saving periodontal therapy.

Many researchers used PRF for supporting wound healing in clinical treatment of gingival recession and implant surgery. Platelet-rich fibrin was used to support the recovery of alveolar bone in implant surgery and this study had obtained encouraging results. In 2006, Choukroun⁴ combined PRF with lyophilized bone allograft for alveolar bone augmentation. Histological evaluation showed the formation of new bone structure and connective tissue surrounding in groups with or without using PRF. However, the time to complete bone structure is 4 months when using PRF and the time was shortened compared to nonuse of PRF which is 8 months. Platelet-rich fibrin created by the process of Choukroun was used in implant surgery, which showed bone regeneration after 6 months of surgery, according to research by Mazar et al.⁶ This study also confirmed that PRF is a simple, inexpensive material that can be used to restructure the alveolar bone in implant surgery. In addition, the PRF can be pressed to create a PRF membrane for the application. Gassling et al⁷ announced that PRF membranes were better than commercial collagen membranes (BioGide®) in compatibility and in supporting the proliferation of periosteal cells, and found them to be suitable as a scaffold in periosteal tissue engineering. In a study of Simonpieri et al,^{8,9} on using PRF membrane combined with freeze-dried bone allograft and 0.5% meronidazole solution in implant

surgery obtained some positive results. Platelet-rich fibrin membrane protects graft materials, reduces infection, and stimulates the gingival tissue formation surrounding the implant, and bone graft materials were not lost over time. Platelet-rich fibrin membrane connects the graft materials, the matrix supporting the formation of new blood vessels, stem cell adhesion, and migration of osteoblasts into the transplant center. After 3 months, the implant can be transplanted. After 6 months of implantation, with the support of the PRF, the implant has been fixed by a thick layer of surrounding gingival tissue and can be fixed only after less than 10 months of treatment. Cosmetic results after treatment remain stable after 4 years. In addition to recovery and regeneration of alveolar bone structure, the PRF also supports in treating gingival recession when combined in treatment therapy. Some researchers applied PRF in the treatment of gingival recession, such as Aroca et al¹⁰ and Anilkumar et al.¹¹ Hafez et al¹² studied the use of PRF membrane to cover the implant immediately after implantation. In this study, autologous bone graft material (from the chin) was combined with fibrin to fill bone defects around the implant; then PRF membrane was used to cover around the implant. The histological evaluation and clinical results showed wound healing of soft tissue around the implant and the bone structure was stable. Platelet-rich fibrin membrane will be easily applied as a material in the process of periodontal healing and regeneration. There are a lot of studies on PRF applications in the treatment of alveolar bone defects, implant restorations, periodontal treatment, and gingival recession. However, there are only a few studies on the mechanism of PRF in stimulating wound healing, especially in stimulating internal stem cell sources related to periodontal regeneration. This research was conducted to evaluate the effectiveness of PRF on the migration and proliferation of human gingival fibroblasts (hGFs).

MATERIALS AND METHODS

Isolation and Culture of Human Gingival Fibroblasts

Human gingival fibroblasts were isolated from explanted gingival connective tissue. Briefly, human explanted gingival connective tissues were collected from healthy adults at the University of Medicine and Pharmacy, Ho Chi Minh City, Vietnam. The gingival tissues were sterilized in phosphate buffer saline (PBS, Gibco) supplemented with antibiotics [500 µg/mL streptomycin (Sigma, USA) and 500 UI/mL penicillin (Sigma, USA)]. The tissues were then washed three times in PBS and cut into 4×4 mm small pieces and cultured in complete medium [Dulbecco's Modified Eagle medium: Nutrient

mixture F-12 (DMEM/F12-Sigma, USA) supplemented with 10% fetal bovine serum (FBS–Sigma, USA), 100 µg/mL streptomycin (Sigma, USA), and 100 UI/mL penicillin (Sigma, USA)]. Human gingival fibroblasts that outgrew from the tissue were harvested for further subculture period. Human gingival fibroblasts at passage 4 (P4) were used for the study.

Preparation of PRF

Blood samples were collected from healthy and nonsmoking volunteers (the age range of 20–30) with no history of aspirin intake or other medications that might interfere with coagulation over the previous 2 weeks. Platelet-rich fibrin preparation was performed according to a previously reported protocol¹³ with modification. Briefly, 10 mL blood was drawn from the antecubital vein in test tube without an anticoagulant and centrifuged immediately (within 2 minutes) by a Hettich Universal 220 machine at 2,500 rpm for 15 minutes. After centrifugation, there were three layers: Platelet-poor plasma (PPP) as a supernatant, a PRF clot in the middle, and a red blood cell (RBC) base at the bottom of the tube. The resulting PRF clots were harvested with sterilized forceps and separated from RBC base using scissors.

Platelet-rich Fibrin Extraction

Platelet-rich fibrin extract was prepared according to the reported method.¹⁴ Briefly, PRF clots formed from each 10 mL blood sample were incubated in 25 mL DMEM/F12 at 37°C for 24 hours. After incubation, PRF clots were compressed using forceps in order to collect the fluid component into the incubated medium. The obtained solution was used as PRF extract for further experiments.

Histological Evaluation

Platelet-rich fibrin clot was cut into small samples and fixed in 10% formalin. The fixed samples were embedded in paraffin and cryo-sectioned in 5 µm slide and stained with hematoxylin and eosin for histological evaluation.

Effect of PRF on Cell Proliferation

To evaluate the PRF's role in cell proliferation, hGFs were seeded in 96-well plate (Corning Life Science) at the concentration of 10^3 cells/well and cultured for 24 hours. Culture medium was replaced by PRF liquid extract and cultured for the next 1, 3, 5, and 7 days to investigate the effect of PRF on cell proliferation. Complete medium and FBS-free medium were used as controls for comparison. At each indicated time points, cells were detached by trypsinization (0.25% Trypsin/ethylenediaminetetraacetic acid – Sigma, USA), stained with Trypan Blue (Sigma,

USA), and counted with hemocytometer to determine the number of cells.

Effect of PRF on Cell Migration

Scratch wound healing assay mimics cell migration during wound healing *in vivo*,¹⁵ which is suitable for studying the effect of PRF on cell migration. This assay was conducted according to a previous publication.¹⁶ Human gingival fibroblasts were seeded into 35 mm Petri dishes (5×10^4 cells per dish) and cultured until 80% confluence. A scrape was produced in the monolayer on each dish using a pipette tip. Nonadherent cells were removed by washing once in PBS. Platelet-rich fibrin extract was added in the dishes and cultured for the next 24, 48, and 72 hours. Cell migration into the empty scraped surface under PRF extract condition was monitored using light microscopy (Olympus, Japan) and compared with cell migration under culture medium condition.

RESULTS

Morphology of hGFs

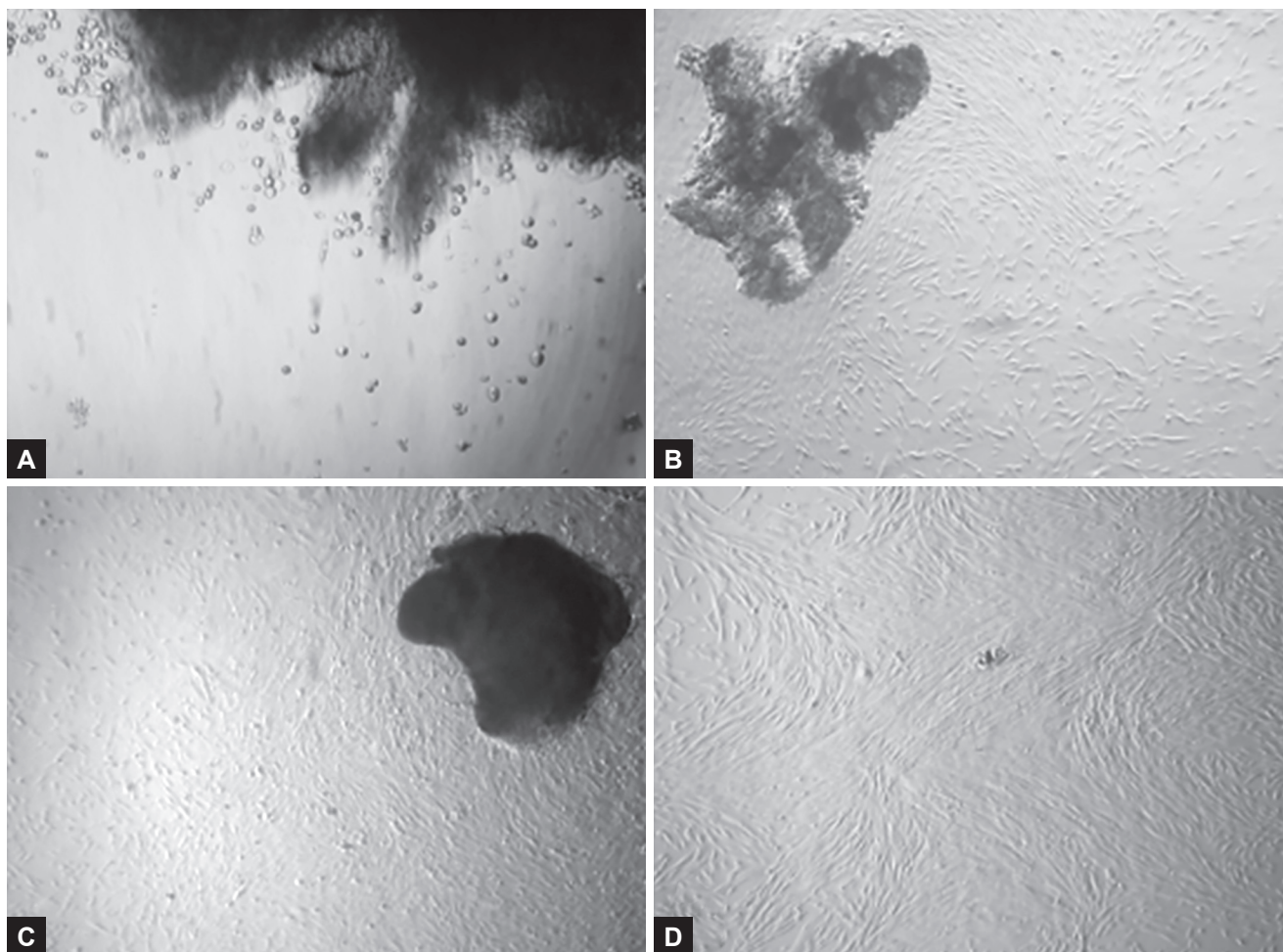
Human gingival fibroblasts were isolated using tissue explant method. Cell migration out from the tissue was observed after 3 days of the primary culture. There were many cell shapes and appearances at the early cultivation period (Fig. 1A). After 5 days of incubation, more cells outgrew from the tissue (Fig. 1B). The cells continued to grow and cover all the culture surfaces in 15 days (Figs 1C and D). Cells proliferated up to 80% confluence and gained homogenous morphology with bipolar and elongated shape which was found to be similar to human fibroblasts in published studies.^{17,18}

Fabrication of PRF

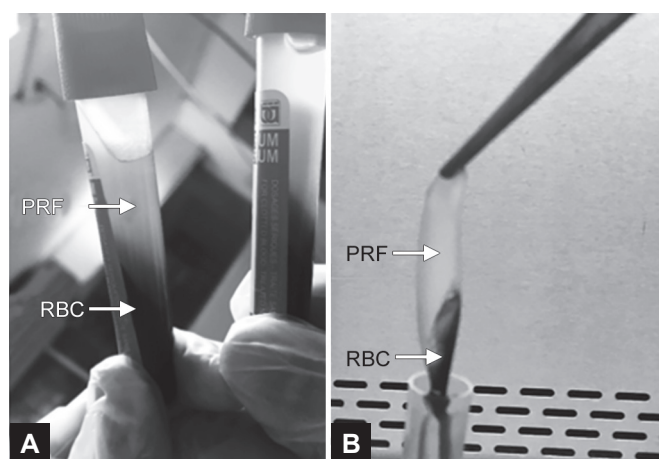
Platelet-rich fibrin was prepared by centrifugation at 2,500 rpm for 15 minutes. Platelet-rich fibrin clot is in the upper phase after centrifugation, whereas RBCs were spun down at the bottom of the tube (Fig. 2A). Platelet-rich fibrin clots were harvested using sterilizing forceps (Fig. 2B). Hematoxylin and eosin staining images visualized a fibrin fiber network architecture (Fig. 3), in which fibrin fibers appeared light pink, whereas RBCs and leukocytes were intensely red and dark blue respectively. Platelets were also dark blue; however, they were recognized as small spots in comparison with leukocytes.

Influence of PRF on Cell Proliferation

The influence of PRF on hGF proliferation was shown as a histogram of the time-dependent cell number (Graph 1). Human gingival fibroblasts maintained the normal



Figs 1A to D: Primary culture of human gingival fibroblast at day 3 (A), day 7 (B), day 10 (C), and day 15 (D). 20× magnification



Figs 2A and B: Platelet-rich fibrin clots after centrifugation. Platelet-rich fibrin clot are in the upper phase and RBCs are at the bottom of the tube

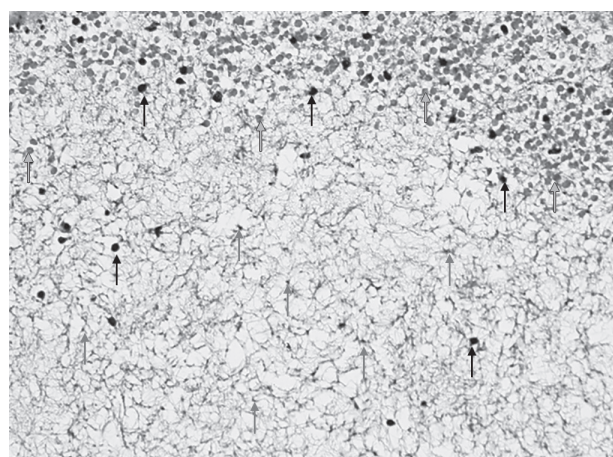
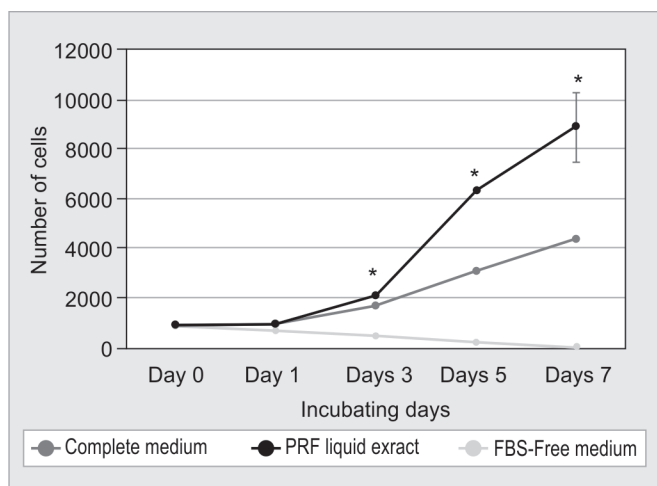


Fig. 3: Hematoxylin and eosin staining of PRF clot. Fibrin fibers are stained pink. Red arrows indicate RBCs. Blue arrows indicate leukocytes. Yellow arrows indicate platelets. 40× magnification

proliferation in complete medium after seeding and 7-day cultivation period indicated by the significant increase in total cell culture yields from days 1 to 7; whereas, there was cell death after extended incubation in FBS-free medium. Human gingival fibroblast treated

with PRF liquid extract represented a significantly better proliferation. Total cell yields in PRF liquid extract group were estimated to be twice higher than those in complete medium group at days 5 and 7. The result indicated that PRF was able to promote the proliferation of hGFs.



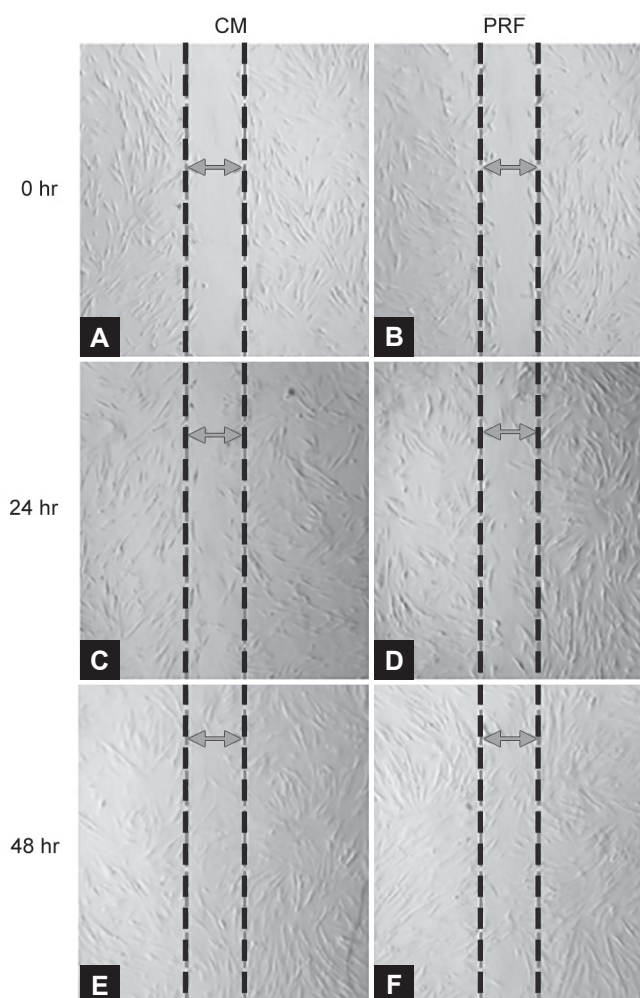
Graph 1: Viability of hGFs cultured in complete medium (blue line), FBS-free medium (yellow line), or PRF liquid extract (red line) from days 0 to 7. *means $p < 0.05$

Influence of PRF on Cell Migration

Scratch wound healing assay was conducted to assess the influence of PRF on cell migration. A scratch was performed on the hGF monolayer (Figs 4A and B). After 24 hours incubation, hGF treated with PRF liquid extract migrated into the scratch area as similar as those treated with complete culture medium (Figs 4C and D). Furthermore, there was no obvious difference in cell migration for both treatments after 48 hours incubation (Figs 4E and F). The result suggested that PRF was able to promote the migration of hGFs.

DISCUSSION

Platelet-rich fibrin which is the second-generation PRP is fabricated according to Choukroun's published protocol without introducing any anticoagulants and foreign factors, such as bovine thrombin or calcium chloride. Therefore, PRF is considered as a natural autologous self-derived biomaterial.^{4,13} Platelet-rich fibrin is a strong natural fibrin matrix, which is similar with the fibrin network in blood clots, and is produced as sealing membranes used in periodontitis treatments. The benefits of PRP in dentinogenesis were demonstrated via its influences on human dental pulp stem cells through cell proliferation and alkaline phosphatase activity.¹⁹ In this study, the potential function of PRF in wound healing was investigated on hGFs for cell proliferation and migration, which would provide evidence for the use of PRF in periodontitis treatments. Our results showed that PRF promoted the proliferation and migration of hGFs. Cell viability was detected to be increasing when extending the periods of time incubation in PRF liquid extract. In contrast, FBS-free medium, which is used for PRF extraction, did not maintain cell growth. Besides, hGFs



Figs 4A to F: Human growth fibroblast migration under PRF liquid extract treatment in comparison with hGFs culture in complete medium. Arrows indicate the range of cell migration

did not only proliferate in PRF liquid extract, but also achieve nearly twice higher amount in comparison with hGFs cultured in complete medium. Therefore, it was strongly demonstrated that PRF could release growth factors under incubated condition and supported cell proliferation. This result is suitable with previous publications about PRF-derived growth factors, such as PDGF-AB, transforming growth factor β , vascular endothelial growth factor, insulin-like growth factor 1.^{1,20}

CONCLUSION

Periodontitis is inflammation of the tissues surrounding the tooth affecting the gingiva, periodontal ligaments and the bone and in its severe forms there can be loss of bone that supports the tooth, resulting in the tooth becoming loose and even causing tooth loss.²¹ The end goal of tissue engineering is to develop products capable of healing diseased or lost tissues and organs. Periodontal regeneration is considered to be organically promising but clinically capricious.²²

Upon the simple protocol modified from Choukroun method, we successfully fabricated PRF and demonstrated its influences on promoting the proliferation and migration of hGFs. From these results, PRF can be applied in clinical periodontitis treatments, especially in treating inflamed gingiva and periodontal tissues. It is expected to provide an appropriate support for wound healing, improve efficiency, and less time consuming. Evidences of PRF effects on hGFs would become a development premise for further research and investigation of PRF on the cells involved in wound healing, and to promote the clinical application of PRF for dental care.

REFERENCES

1. Ross R, Glomset J, Kariya B, Harker L. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells *in vitro*. *Proc Natl Acad Sci USA* 1974 Apr;71(4):1207-1210.
2. Nevins M, Giannobile WV, McGuire MK, Kao RT, Mellonig JT, Hinrichs JE, McAllister BS, Murphy KS, McClain PK, Nevins ML, et al. Platelet-derived growth factor stimulates bone fill and rate of attachment level gain: results of a large multicenter randomized controlled trial. *J Periodontol* 2005 Dec;76(12):2205-2215.
3. Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. Platelet-rich plasma: growth factor enhancement for bone grafts. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1998 Jun;85(6):638-646.
4. Choukroun J, Diss A, Simonpieri A, Girard MO, Schoeffler C, Dohan SL, Dohan AJ, Mouhyi J, Dohan DM. Platelet-rich fibrin (Prf): a second-generation platelet concentrate. Part V: Histologic evaluations of PRF effects on bone allograft maturation in sinus lift. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006 Mar;101(3):299-303.
5. Naik B, Karunakar P, Jayadev M, Marshal VR. Role of platelet rich fibrin in wound healing: a critical review. *J Conserv Dent* 2013 Jul-Aug;16(4):284-293.
6. Mazor Z, Horowitz RA, Del Corso M, Prasad HS, Rohrer MD, Dohan Ehrenfest DM. Sinus floor augmentation with simultaneous implant placement using Choukroun's platelet-rich fibrin as the sole grafting material: a radiologic and histologic study at 6 months. *J Periodontol* 2009 Dec;80(12):2056-2064.
7. Gassling V, Douglas T, Warnke PH, Acil Y, Wiltfang J, Becker ST. Platelet-rich fibrin membranes as scaffolds for periosteal tissue engineering. *Clin Oral Implants Res* 2010 May;21(5):543-549.
8. Simonpieri A, Del Corso M, Sammartino G, Dohan Ehrenfest DM. The relevance of Choukroun's platelet-rich fibrin and metronidazole during complex maxillary rehabilitations using bone allograft. Part I: a new grafting protocol. *Implant Dent* 2009 Apr;18(2):102-111.
9. Simonpieri A, Del Corso M, Sammartino G, Dohan Ehrenfest DM. The relevance of Choukroun's platelet-rich fibrin and metronidazole during complex maxillary rehabilitations using bone allograft. Part II: implant surgery, prosthodontics, and survival. *Implant Dent* 2009 Jun;18(3):220-229.
10. 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. *J Periodontol* 2009 Feb;80(2):244-252.
11. Anilkumar K, Geetha A, Umasudhakar, Ramakrishnan T, Vijayalakshmi R, Pameela E. Platelet-rich-fibrin: a novel root coverage approach. *J Indian Soc Periodontol* 2009 Jan-Apr;13(1):50-54.
12. Hafez WK, Seif SA, Shawky H, Hakam MM. Platelet rich fibrin as a membrane for coverage of immediate implants: case-series study on eight patients. *Tanta Dent J* 2015 Sep;12(3):203-210.
13. Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJ, Mouhyi J, Gogly B. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part I: Technological concepts and evolution. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006 Mar;101(3):e37-e44.
14. Ji B, Sheng L, Chen G, Guo S, Xie L, Yang B, Guo W, Tian W. The combination use of platelet-rich fibrin and treated dentin matrix for tooth root regeneration by cell homing. *Tissue Eng Part A* 2015 Jan;21(1-2):26-34.
15. Rodriguez LG, Wu X, Guan, JL. Wound-healing assay. *Methods Mol Biol* 2005;294:23-29.
16. Li R, Guo W, Yang B, Guo L, Sheng L, Chen G, Li Y, Zou Q, Xie D, An X, et al. Human treated dentin matrix as a natural scaffold for complete human dentin tissue regeneration. *Biomaterials* 2011 Jul;32(20):4525-4538.
17. Damante CA, De Micheli G, Miyagi SP, Feist IS, Marques MM. Effect of laser phototherapy on the release of fibroblast growth factors by human gingival fibroblasts. *Lasers Med Sci* 2009 Nov;24(6):885-891.
18. Hakki SS, Bozkurt SB. Effects of different setting of diode laser on the mRNA expression of growth factors and type I collagen of human gingival fibroblasts. *Lasers Med Sci* 2012 Mar;27(2):325-331.
19. Huang FM, Yang SF, Zhao JH, Chang YC. Platelet-rich fibrin increases proliferation and differentiation of human dental pulp cells. *J Endod* 2010 Oct;36(10):1628-1632.
20. Zumstein MA, Berger S, Schober M, Boileau P, Nyffeler RW, Horn M, Dahinden CA. Leukocyte- and platelet-rich fibrin (L-Prf) for long-term delivery of growth factor in rotator cuff repair: review, preliminary results and future directions. *Curr Pharm Biotechnol* 2012 Jun;13(7):1196-1206.
21. Saini R. A Prospective Experimental Comparative Study on the Clinical Effects of Calculus Dissolution based Oral Rinse in Gingivitis Patients. *Int J Experiment Dent Sci* 2015;4(1):33-39.
22. Saini R, Saini S, Sharma S. Therapeutics of stem cells in periodontal regeneration. *J Nat Sc Biol Med* 2011;2:38-42.