

Original Article

The Effect of Platelet-Rich Fibrin (PRF), Plasma Rich in Growth Factors (PRGF), and Enamel Matrix Proteins (Emdogain) on Migration of Human Gingival Fibroblasts

Mohammad Reza Talebi Ardakani, DMD, MScD¹; Mansour Meimandi, DMD, MScD²; Reza Shaker, DMD, MScD³; Shima Golmohammadi, DMD, MScD⁴

¹ Dept. of Periodontics, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

² Dept. of Periodontology, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

³ Periodontist, Private Practice, Tehran, Iran.

⁴ Dept. of Periodontology, Islamic Azad University of Borujerd, Borujerd, Iran.

KEY WORDS

Platelet-rich plasma;
PRF;
Enamel matrix proteins;
Fibroblast;
Wound Healing;

Received: April 2018;
Revised: October 2018
Accepted: December 2018;

ABSTRACT

Statement of the Problem: Platelet preparations such as plasma rich in growth factors (PRGF), platelet-rich fibrin (PRF), or enamel matrix proteins (Emdogain) are commonly used for soft and hard tissue regeneration. However, their comparative effectiveness has not been extensively studied, and a consensus has yet to be reached on their efficacy.

Purpose: The aim of this *in vitro* study was to compare the effect of PRF, PRGF, and Emdogain on the proliferation of human gingival fibroblasts (HGF).

Materials and Method: Artificial wounds were made in HGF cell culture after the fibroblasts reached nearly 100% of confluence. The study groups included leukocyte and platelet-rich fibrin (LPRF), pure platelet-rich fibrin (PPRF), PRGF and Emdogain. Stained cells were photographed at 48 h and one week and the percent of wound filling was measured. Statistical analysis was performed by one-way ANOVA and *p* values < 0.05 were considered statistically significant.

Results: The highest wound filling percentages at both time intervals were observed in the PPRF group, followed by the PRGF. The lowest percentage of wound healing among test groups was observed in the LPRF while Emdogain yielded modest results. However, statistical analysis showed similar wound healing values in PRGF, PPRF, and Emdogain groups.

Conclusion: Within the limitations of this study, PRGF, PPRF, and Emdogain were similarly effective in enhancing the fibroblast proliferation and artificial wound closure.

Corresponding Author: Golmohammadi Sh, Dept. of Periodontics, Islamic Azad University of Borujerd, Borujerd, Iran. Tel: +986633335303 Fax: +986633120149 Email: shimag221@gmail.com

Cite this article as: Talebi Ardakani MR, Meimandi M, Shaker R, Golmohammadi Sh. The Effect of Platelet-Rich Fibrin (PRF), Plasma Rich in Growth Factors (PRGF), and Enamel Matrix proteins (Emdogain) on Migration of Human Gingival Fibroblasts. *J Dent Shiraz Univ Med Sci.* December 2019; 20(4): 232-239.

Introduction

The final aim of periodontal therapy is to establish a healthy periodontium and, if achievable, restore lost form and function [1]. Healing of periodontium requires the balanced involvement of gingival connective tissue, periodontal ligament, cementum, and bone [2]. Final appearance and function of tissue may be influenced by different factors affecting the healing process. On the other hand, the final tissue can be a non-functional fibrous (scar) tissue, partially functional repaired tissue or

a completely regenerated functional tissue [3].

In the ideal healing process, all the components of attachment apparatus are restored similar to the course of normal development [4]. However, it has been shown that periodontal wound healing after conventional periodontal therapy most commonly leads to the formation of a fibrous tissue and apical migration of gingival epithelium between the gingival connective tissue and the root surface. This healing process does not constitute regeneration [5].

Gingival fibroblasts are the dominant cells in the healthy gingival connective tissue and play an important role in its development by maintaining the composition and structural integrity of the extracellular matrix. Fibroblasts have also remodeled collagen during the connective tissue healing [6]. After tissue injury, wound healing requires the presence of cells with regenerative capacity at the healing site. In this phenomenon, fibroblasts should be able to stabilize and start matrix formation. On the other hand, adhesion of fibroblasts to extracellular matrix is of special importance in maintaining proper cell morphology, cell function, and tissue integrity [7]. Therefore, regarding the influence of biologic agents on cellular activity, the gingival fibroblast cell line is of main importance. Considering their relative advantages, they can be easily isolated from the patients and can grow fast in a normal culture medium [8].

As originally proposed by Slavkin and Boyde [9], enamel matrix proteins are known to have the ability to regulate the mineralization of enamel [10] and they are released by the epithelial cells during root formation and can affect the cementogenesis and the formation of supporting periodontal tissues [11].

Emdogain is the brand name for porcine enamel matrix derivatives (EMD) and has been successfully used for regeneration of periodontal tissues in patients suffering from periodontal disease [12-13]. Emdogain has been clinically applied for regeneration of intra-bony defects, furcations, and gingival recessions. The *in vivo* studies suggested that EMD somehow restricts the down-growth of the oral epithelium into regenerating periodontal defects [14]. However, it enhances the proliferation and differentiation of cultured periodontal ligament (PDL) cells [15]. The *in vitro* effects of Emdogain have been studied on various cell systems indicating that Emdogain promotes the differentiation of osteogenic precursors, enhances the proliferation and matrix production of PDL cells, but inhibits the proliferation of epithelial cells. The study by Zeldich *et al.* [16] explained the cellular pathway for the mitogenic effect of EMD on human gingival fibroblasts (HGFs).

Platelet concentrates are products obtained after processing the whole blood, mostly through centrifugation. There are custom-made systems or many techniques commercially marketed for production of platelet con-

centrates among which plasma rich in growth factors (PRGF) is broadly presented. PRGF includes several growth factors and bioactive proteins that facilitate the process of wound healing and tissue regeneration [17]. Several researchers have indicated improved recovery and new bone formation following the use of PRGF [18-19]. However, such results have not been reported in some other studies [2, 20].

Pure platelet-rich fibrin (PPRF) is by definition a preparation without leukocytes with a high-density fibrin matrix. The fibrin matrix per se has significant effects on the differentiation of osteoblasts since it traps the platelets and cytokines, which are probably released after a certain period [21].

Leukocyte and platelet-rich fibrin (LPRF) is the second-generation platelet concentrate introduced for use in oral and maxillofacial surgeries and tissue repair. It is obtained by immediate centrifugation of whole blood without anticoagulants. Quick activation of the coagulation cascade and formation of thrombosis during centrifugation induce fibrin formation and platelet activation. Due to the use of the mechanical process of polymerization during centrifugation, composition and quantity of growth factors in LPRF clots remain stable. The LPRF membrane slowly releases high volumes of growth factors such as transforming growth factor-beta 1 (TGF β -1), platelet-derived growth factor AB (PDGF-AB), vascular endothelial growth factor (VEGF) and other matrix glycoproteins for at least seven days [22-23].

In the process of natural healing, platelets aggregate and release growth factors [24]. The results of a study by Tuan *et al.* [25] on the role of fibrin in tissue healing showed that fibroblasts have the ability to reconstruct a fibrin matrix and initiate collagen production. In this respect, it appears that platelet-rich fibrin (PRF) compact fibrin matrix plays a role in the stimulation of wound healing process. Additionally, PRF can lead to a controlled release of growth factors over time [26] as the levels of TGF β -1 and PDGF-AB in the PRF increase up to day 14 and decrease afterward. LPRF can provide a long-term release of growth factors and maintains the potential to cause a delay in maximum release values [17].

For further clarification of the mechanism underlying the observed *in vivo* effects of these biologic prod-

ucts, the present study was designed to examine and compare the effects of PPRF, LPRF, PRGF, and Emdogain on gingival fibroblasts.

Materials and Method

Preparation of PRF and PRGF

Blood samples were collected from a 30-year-old, non-smoker, healthy volunteer after obtaining her written informed consent. To prepare LPRF, 27ml of the venous blood was poured into three dry blood collecting tubes (Blood collecting tubes[®], Process, Nice, France) without anticoagulants. According to Choukroun's protocol, the tubes were immediately centrifuged at 2700 rpm for 12 minutes (approximately 400 g) and a compact fibrin clot was formed in the middle of the tube in between the red blood cell (RBC) layer at the bottom and plasma poor in platelet (PPP) fraction at the top. PRF box was employed in a sterile environment to obtain standard PRF membranes. To prepare PPRF using the Fibrinet[®] (Cascade Medical Enterprises; LLC, Wayne, NJ, USA), uncoagulated blood was collected into a tube containing a thixotropic separator gel to isolate plasma and platelets without the leukocytes and RBCs. After the first spin at 1100 g for 10 min, the second phase of centrifuge was performed at 1500 g for 15 min in tubes containing CaCl₂.

For PRGF, twenty-seven milliliters of venous blood were poured into 9ml sterile blood collecting tubes (BTI) containing 0.5ml of the anticoagulant agent (3.8% sodium citrate). According to the standard technique (BTI PRGF[®] System III; Biotechnology Institute, S.L., Álava, Spain), the blood sample was first centrifuged at 1800 rpm for 8 minutes and consequently the blood was separated into 4 fractions of plasma poor in growth factors (PPGF) at the surface of the tube (1 ml), plasma containing growth factors (0.5 ml), PRGF, immediately above the RBCs (0.5 ml), and RBCs. The PRGF layer was used in this study. PRGF was separated by 0.5 ml pipettes and transferred to other tubes. Subsequently, 0.05 ml of 10% calcium chloride was added for every 1 ml of the PRGF to activate it. Ten minutes later, the obtained gel was added to the plates containing fibroblast cells. Emdogain was obtained from the manufacturer (Straumann, Mehrarabon Co. Iran).

Cell culture and treatment

Human gingival fibroblast HGF-1 cell line was obtained

from the National Cell Bank of Iran (NCBI code: C165, Pasteur Institute, Tehran, Iran). The fibroblast cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), and 1% antibiotic-antimycotic (2mg/ml glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 12.5 U/ml nystatin, 0.11 mg/ml sodium pyruvate and nonessential amino acids), and incubated at 37 °C for 2 hours in CO₂ incubator. Afterward, the subconfluent growing cells were plated in 60 mm Petri dishes, which were coded. The total number of the dishes, which contains the culture medium supplemented with the test materials, was 18.

The Petri dishes containing 10% and 1% FBS was considered as positive and negative control groups, respectively.

After the monolayer cells reached near 100% confluence, a series of cells were detached in the marked path using a cell scraper. By doing so, an artificial wound of approximately similar size was created. In the next phase, cells were rinsed with the culture medium several times in order to wash off the detached cells. The cell Petri dish was evaluated under a microscope on a daily basis. At the specified time points, cells were fixed and stained with crystal violet. In order to obtain the same field during imaging, markings were made with an ultra-fine tip marker on the outer bottom of the dishes located on both sides of the scratches. After the reference points were made, stained cells were digitally photographed under the same exposure conditions, and the percent of wound filling in the presence of Emdogain, PRGF, LPRF, and PPRF were determined at 48 h and one week, using Image J software.

Statistical analyses

The assay was performed in triplicate for each group. The mean percentage of wound filling in each group was calculated and intergroup comparisons were performed using one-way ANOVA. P-values of less than 0.05 were considered as statistically significant.

Results

An image of stained cells at one week is shown in Figure 1. The percentage of wound filling in PRGF, LPRF, PPRF, Emdogain, 10% FBS (positive control group) and 1% FBS (negative control group) at 48h and one week is shown in Figure 2. The wound filling percentage was significantly improved in all the test groups from 48h to

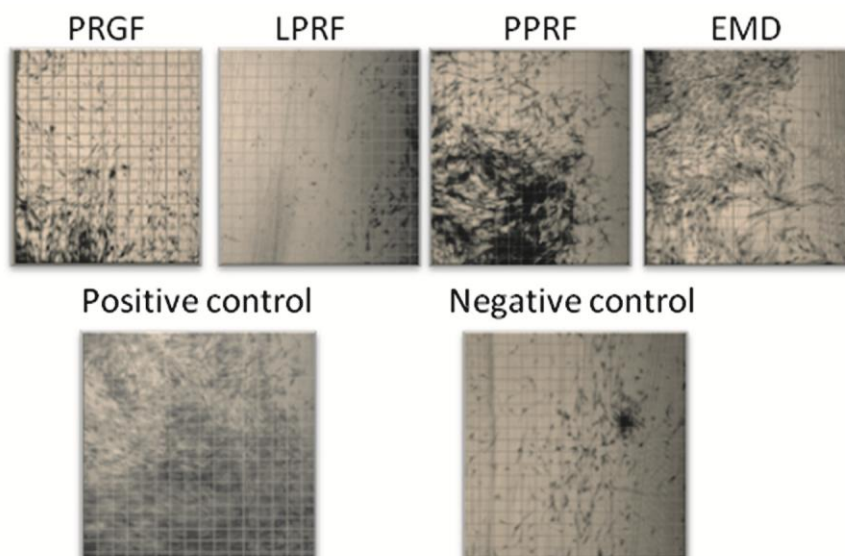


Figure 1: Microscopic analysis of HGFs in four tests and two control groups at one week

one week, ($p < 0.05$). At 48h, the percentage of wound filling was approximately the same in PPRF, Emdogain, and positive control groups ($p > 0.05$). One week after the artificial wound was made; fibroblasts in the PPRF group covered a more percentage than the other test groups. However, this difference was not significant and PRGF, PPRF, and Emdogain had almost a similar percentage of wound filling ($p > 0.05$). The lowest wound coverage was observed in LPRF at both time intervals.

Discussion

Cell proliferation and migration are an essential part of wound healing that may be simply assessed through wound healing/scratch assay [27]. The present study evaluated the effects of PRGF, PRF, and Emdogain on the proliferation of human fibroblasts in filling the artificial wounds. Based on the obtained results, except for 10% FBS as the positive control with the highest percentage of wound filling and LPRF and 1% FBS as the

negative control with relatively small values, the remaining groups were almost in the same range in terms of wound filling percentage. Furthermore, the filling percentage increased over time in all groups.

PRGF is biocompatible, effective, and safe and is absorbed by the body after local regeneration, within a couple of days [18, 28-29]. Application of PRGF as an autologous factor provides an opportunity for the activity of several growth factors and is associated with a possible increase in tissue angiogenesis. Another advantage of using platelet and plasma-based bioactive molecules present in PRGF is their capacity and ability to reduce post-operative tissue inflammation. It has been specified that platelet products are able to inhibit the release of monocyte cytokines and decrease the inflammation [30]. Additionally, platelets are able to inhibit the release of IL-1 primarily from the activated macrophages. This primary inhibition of inflammatory responses may explain the mechanisms through which

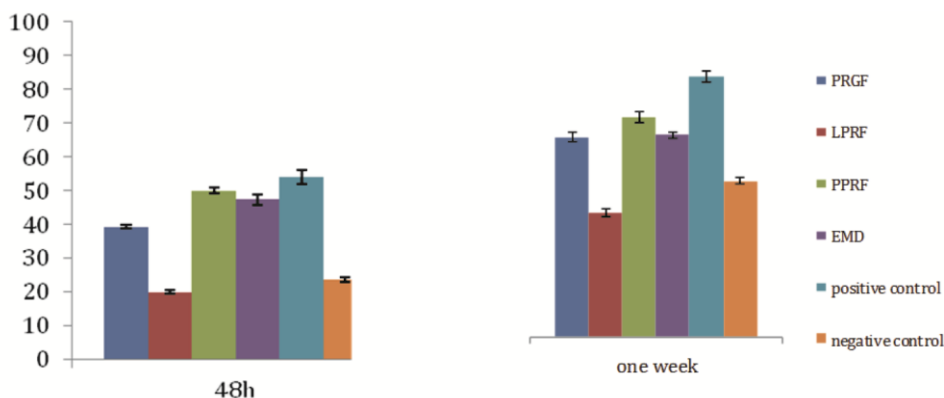


Figure 2: Percentage of wound filling in tests and control groups at 48h and one week

platelet-rich products act as anti-inflammatory agents [31]. Creeper *et al.* [32] assessed the effects of platelet-rich plasma on the migration, proliferation, and differentiation of PDL cells and human osteoblasts under *in vitro* conditions and demonstrated that platelet-rich plasma had the ability to enhance cell function associated to wound healing.

Vahabi *et al.* [33] reported that the application of PRGF caused a significant increase in proliferation of cells at 24, 48 and 72 hours in comparison to 1% FBS and 10% FBS. While, PRF led to a significantly increased proliferation in the test group only at 24 h compared to the control group. It had converse effects on cell proliferation at 48 and 72 h. This was in contrary to the results of a study by Dohan *et al.* [34] that revealed the stimulatory effect of PRF membrane on HGF proliferation in 3, 7, 14 and 21 days compared to the control group. Likewise, in the present study, although not statistically significant, PPRF induced more proliferation and resulted in more wound filling percentage than PRGF at both 48 h and one week. However, the method of our study was different. In the current study, we used *in vitro* scratch assay, which is a straightforward and economical method to study cell migration *in vitro* and evaluates both proliferation and migration of cells along the artificial wound space [27]. The controversial results might also be attributed to the differences in platelet count, the time interval between the centrifugation of blood samples and PRF preparation and its exposure to the culture medium. According to a report by Liu *et al.* [35], cell proliferation rate is pH-dependent and higher platelet concentrations lead to a change in pH, which has a negative effect on fibroblast proliferation. The platelet concentration in PRGF is two or three times more than the normal level, which is ideal for cell proliferation [36-37]. Since the platelets are entrapped in the compact LPRF mesh, it is not feasible to count the number of platelets in LPRF [38]. In this study, PRF and PRGF were obtained from the same donor to eliminate any possible confounding effect caused by inter-individual variations. In addition to the number of platelets, their function in creating a fibrin gel is important in stimulating biological mechanisms [39-40]. Accordingly, their effect on cell growth and proliferation is better to be examined as a whole element rather than separate components [33]. The minimum diameter of culture

plates was 60 mm, which avoids the compression of cultivating cells while adding the fibrin clot is 60 mm [41].

In our study, adding LPRF to the culture medium resulted in the lowest percentage of wound filling, even lower than the negative control. This finding might be explained by deleterious biological effects of matrix metalloproteinases 8 and 9 produced by neutrophils [28]. In addition, neutrophils may induce tissue damage by releasing reactive oxygen species in the inflammatory phase of tissue injury [42]. However, some authors emphasize on the potential ability of leukocytes in proliferation, differentiation, and immunity [43]. To date, no consensus has been reached on the inclusion of leukocytes in platelet products, and further investigation is required to determine the effects of leukocytes completely. Accordingly, until confirmation of this issue in future studies, use of platelet products lacking leukocytes is more prudent.

The mitogenic activity of the platelet supernatant is not limited to its growth factor content [40]; whereas; microparticles and platelet membrane fragments play a significant role under *in vitro* conditions [4]. Except for few studies [34, 41], other laboratory studies evaluated the effects of PRP and PRF supernatant and this issue is an important limitation of our study. Since we only assessed the growth factors and proteins presented in the exudate of these two materials under the mentioned conditions, the actual effects of the whole products were disregarded. PRF is a compact fibrin with a unique matrix structure and cell content and its exudate is not the only derivative of this product. Furthermore, although PRP is a homogenous liquid product, its exudates are not the only present active component and aggregation of platelets for fibrin gel formation stimulates many biological mechanisms [39-40].

Mitogenic effects of Emdogain on HGF have been reported in a number of studies [45-46]. Zedlich *et al.* [16] demonstrated that application of 100 µg/ml Emdogain increased collagen production by gingival fibroblasts. Stimulation of these cells by the EMD was also associated with increased proliferation and production of extracellular matrix. Sanders *et al.* [47] evaluated the effects of varying concentrations of EMD with and without cyclic mechanical strain on cellular wound healing and wound fill of human fibroblasts and re-

vealed that percentage of wound fill ranged from 34.1% to 55.7% in zero to 120 µg/ml concentrations of Emdogain. At higher concentrations, Emdogain had no significant effects on the percentage of wound fill in controlling the gingival fibroblasts.

It is clear that *in vitro* experiments are helpful for assessing the biological efficacy of blood products, but these studies have limitations in mimicking the clinical conditions. Thus, it may be unrealistic to generalize *in vitro* findings to *in vivo* situations. Environmental differences between the laboratory and the real conditions of the wound healing process may affect the cellular interactions. Saliva contains numerous cytokines, growth factors, and protease inhibitors that are key factors for fast healing. However, these are not the only factors responsible for the quick healing of the oral mucosa. Only large palatal wounds may be affected by the absence of saliva, and smaller wounds are healed normally. Although saliva can be helpful in the process of healing, it seems unlikely that it is completely responsible for scarless healing of the oral mucosa. There is a possibility that intrinsic properties of oral mucosa play a critical role in the healing of oral tissues.

Conclusion

PRGF, PPRF, and Emdogain had relatively similar efficacy in enhancing fibroblast proliferation and artificial wound closure.

Conflict of Interest

None declared.

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