

Comparison of Enamel Matrix Derivatives and Platelet-Rich Fibrin on Human Gingival Fibroblasts Proliferation and Viability

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Introduction: Biologic mediators such as platelet-rich fibrins (PRF) or enamel matrix derivatives (Emdogain®) are used for the regeneration of the soft and hard tissues. The present study aimed to compare the efficacy of PRF and Emdogain® on the proliferation and differentiation of gingival fibroblasts in vitro. **Materials and Methods:** In this randomized controlled in vitro trial, blood samples were obtained from a healthy person and Choukroun's PRF was prepared according to the standard protocol. Fibroblast cells were cultured with PRF and different concentration of Emdogain®. MTT test were used to measure fibroblast cells proliferation after 24 and 72 hours. **Results:** As compared to negative control (FBS 1%), Emdogain® 50µg/ml significantly increased cells proliferation up to 123.2% and 111.2% after 24 and 72 hours. The values were 214.9% and 121.9% for Emdogain® 100µg/ml. The increased proliferation values were significant for both groups after 24 hours ($P<0.001$) while the values were significant for 100 µg/ml Emdogain after 72 hours ($P<0.001$). Cells exposure to PRF showed significant effect on their proliferation after 24 hours compared to the negative controls ($P<0.001$; $21\pm1.73\%$). After 72 hours; PRF showed reverse effect on the proliferation and decreased them up to 38% and 60% regarding negative controls. **Conclusion:** the most proliferation and viability of fibroblast cells were obtained after exposure to Emdogain® and the differences between Emdogain® and PRF were significant.

Keywords: Platelet-rich Fibrins ; Emdogain; Gingival Fibroblasts; Proliferation; Viability

Introduction

The final purpose of periodontal treatments is the regeneration of periodontal tissues lost during the disease process (1, 2). Until the mid-1970s, the major strategy for achieving this purpose was using resective and reconstructive, which were associated with several issues, such as esthetic, root sensitivity after surgery, and increased risk of root caries (3, 4). The efficacy of these techniques in preventing the progression of periodontal disease has been proven (5); Periodontium healing requires balance of the gingival connective tissue, periodontal ligament, cementum, and bone (6). Subsequently, many efforts have been made to regenerate periodontal tissues and many methods and techniques have been introduced (7). In the 1990s, enamel matrix proteins (EMPs) emerged as a biological material for periodontal tissue regeneration. Enamel matrix derivatives (EMD) contains proteins that are thought to play a key role in the development of the tissues surrounding and has been found to favour cutaneous wound healing (8, 9). It was named

Emdogain® and first introduced in 1996 by a company in Switzerland (Biora). In 2003, Biora joins Straumann as its subsidiary and since then, its name has been to the Straumann (9). The current EMP (Emdogain®) contains EMD and PGA (polyglycolic acid) as carriers. It contains hydrophobic enzyme matrix proteins and mainly Amelogenin as the major protein extracted from pig enamel (10). During tooth development, EMP is secreted by ameloblast cells to regulate mineralization (11). EMP is released from epithelial cells during root formation and affects cementogenesis and the formation of supporting tissues around the tooth (12). One of the main properties of EMP is its proliferative effect that has used in in vitro studies on periodontal ligament cells (13). On the other hand, some studies have suggested the direct effect of Emdogain® on gingival fibroblasts (14). Emdogain® has recently been shown to increase the proliferation of gingival fibroblast cells in the rat and humans and prolong the survival time of these cells in the culture medium, despite the presence of an apoptotic stimulus (15-17). Numerous in vitro studies have been performed to

elucidate the mechanism of the effect of Emdogain® on different cell types, however, its molecular mechanism is still unknown (18). Emdogain® is currently used successfully for the regeneration of periodontal tissues lost following periodontal disease (19). In addition, it is used to take root cover with the coronally advancement flap (14). Platelet-Rich Plasma (PRP) has been widely studied to achieve tissue regeneration. It was first introduced to the medical community by Whitman et al. (1997) as the platelet gel (20). However, the use of PRP has been suggested for periodontal regenerative procedures (21). It has used in medicine, especially among oral and maxillofacial surgeons. Using biological mediators, especially Emdogain® and PRP to achieve a faster wound healing and tissue regeneration is developing (22). This issue is also performed in clinical situation for intrabony defects (23-25). Due to the not enough information regarding their properties and the superiority under similar conditions, they should be compared. The aim of this study was to determine the effects of Emdogain® and PRF on the proliferation and differentiation of gingival fibroblast cells *in vitro*.

Materials and Methods

The study was carried out in the Department of Cellular and Molecular Research, Faculty of Dentistry, Shahid Beheshti University of Medical Sciences. The samples of this experimental study consisted of gingival fibroblasts prepared from the Pasteur Institute of Iran cell bank (HGF.1-PI, NCBI: C-165). Considering the considered sample size of other studies and also due to research conditions, 3 samples were considered for the experimental and control groups. The non-probability sampling (convenience) was done, but the samples were randomly assigned to different groups. Emdogain® was purchased from the Straumann Company (Basel, Switzerland) and a PRF sample was obtained from an eligible subject after obtaining consent.

After obtaining consent form, the blood sample was collected from a healthy volunteer by laboratory technician. 27 ml of venous blood was collected in three 9 ml collecting tubes (Nice, France,) with no anticoagulant. According to the Choukran protocol, the tubes were centrifuged immediately at 700 g (2700 rpm) for 21 min resulted in a dense fibrin clot at the center of the tube between the blood cells on the floor and the above fluid serum. The PRF clot under the sterile hood was cut off by scissors from the blood cells (Figure 1).

Then, the PRF box (PRF box, Process, Nice, France) was used to standardize the samples (Figure 2). Human gingival fibroblast (HGF) cells were obtained from the Pasteur Institute of Iran cell bank. They were placed in DULBECCO'S MODIFIED EAGLE MEDIUM (DMEM) containing 10% FBS and antibiotic (1% Penicillin-streptomycin; Sigma) and cultured at 37°C with 5% CO₂ and 95% O₂. The cell culture solution was replaced every 3 days and cells were used after the fourth passage. Cells were cultured for each test time (24 and 72 h) in Petri dishes (60 ml) including 50,000 cells per petri dish in a final volume of 3.5 ml. They were then placed in medium containing FBS% 2 plus Emdogain® 100µg / mL, FBS% 2 plus Emdogain® 50µg / mL, Choukran's PRF, 10% FBS (positive control), and 1% FBS (negative control) followed by placing in an incubator. In this study, MTT assay was used to evaluate the proliferation of HGF (fibroblast) cells. Accordingly, 24 h after culture, the supernatant was gently removed and the medium containing 1% FBS serum was added to each petri dish to stop cell growth and entering the G0 phase of the cell cycle by the cell. 24 h later, the considered intervention was performed on the cells and they were again incubated. So that the media on Petri dishes was gently removed and then added to 9 Petri dishes containing 1% FBS serum (negative control), 9 Petri dishes containing 10% FBS serum (positive control), 9 Petri dish containing PRF membrane, 9 Petri dish including Emdogain® (50µg / mL) plus 2% FBS, and 9 Petri dish including Emdogain® (100µg / mL) plus 2% FBS (Figure 3). After incubation (24 h for the first set and 72 h for the second set), the Petri dishes were gently removed and cells were washed with approximately 3 ml of PBS buffer. Then, a serum-free medium containing 10% MTT yellow solution (5mg / ml) was added to each petri dish at a final concentration of 0.5mg / ml at room temperature and incubated for 4 h in dark to form purple crystals of formazan. After incubation and confirmation of crystals formation under the microscope, the medium containing MTT was slowly evacuated from each petri dish and then the DMSO solution was added to each dish followed by placing in a shaker for 10 min until the crystals were completely dissolved (Figure 4 and 5). The plate was transferred onto a plate reader and the optical absorption was measured at 570 nm (620 nm as the reference wavelength) (Figure 6). The mean absorption of 9 wells for each treatment was averaged and compared with controls.

In this study, platelet-rich fibrin was obtained from a volunteer who was fully informed about the research objective



Table 1. Percentage of cell proliferation in different groups at 24 and 72 h

Time	Group	Mean cell proliferation as%
Both time points	Negative control	100
24 h	PRF	121.4
24 h	Emdogain®50	123.2
24 h	Emdogain®100	214.9
24 h	Positive control	144.1
72 h	PRF	62.3
72 h	Emdogain®50	111.2
72 h	Emdogain®100	121.9
72 h	Positive control	165.8

after obtaining the written informed consent. HGF cells were also obtained from a valid cell bank and all the principles of and concepts of hygiene and health were observed in laboratory analysis.

Repeated Measure ANOVA was used to compare the effect of Emdogain® and PRF on the proliferation of HGF cells. Due to the significant difference between the groups, Tukey's multiple comparison test was employed. P values of less than 0.05 were considered statistically significant.

Results

To assess the results of the MTT assay, the viability negative control medium (FBS 1%) was considered 100%. Then, the mean optical density values of the studied groups were expressed as a percentage of the control group. The microscopic effects of PRF and Emdogain® on the proliferation of HGF cells at 24 and 72 h are shown in Tables 1 and 2.

According to the results, the cells cultivated in the presence of Emdogain® (50µg/ml) showed an increase of 123.2% and 111.2% in proliferation at 24 and 72 h, compared with the negative control (1% FBS), respectively. These values were obtained 214.9 and 121.9 for Emdogain® (100µg/ml), respectively. This increase in cell proliferation was significant for both groups at 24 h ($P<0.001$), however at 72 h, only the Emdogain® (100µg/ml) showed a significant increase ($P<0.001$).

The results of HGF cells cultivated in the presence of PRF showed a statistically significant effect on the proliferation of cells at 24 h compared with the negative control group (21 ± 1.73 ; $p<0.001$). However, at 72 h, PRF showed an opposite effect, with a 38% and 60% decrease in viability and proliferation of HGF compared with the negative control group, respectively.

Table 2. Fibroblasts proliferation rate in different groups at 24 and 72 h

Time	Groups	No.	Mean±SD	Std. error
24 h	Negative control	9	0.025 ±0.002	0.0009
24 h	Positive control	9	0.037 ±0.004	
24 h	Emdogain50µg/ml	9	0.031±0.004	0.0147
24 h	Emdogain100µg/ml	9	0.054±0.009	0.003
24 h	PRF	9	0.030±0.006	0.0021
72 h	Negative control	9	0.026±0.006	0.002
72 h	Positive control	9	0.043±0.004	0.0151
72 h	Emdogain50µg/ml	9	0.029 ±0.002	0.0007
72 h	Emdogain100µg/ml	9	0.032 ±0.003	0.0009
72 h	PRF	9	0.013±0.002	0.0026

The results of this study showed the highest proliferation and viability of fibroblast cells cultivated in the presence of Emdogain® and the difference between PRF and Emdogain® groups was statistically significant ($p<0.001$).

Discussion

The aim of this study was to investigate the effect of Choukroun's PRF and Emdogain® at two different concentrations on the proliferation of HGF cells. The PRF was introduced as the second generation of Platelet-Rich Plasma in 2001 and considering its fibrin network, has unique and distinct properties (22). The results of PRF showed a significant increase only at 24 h compared with the control group and followed by a reverse effect on cell proliferation. The results of the present study are not consistent with those of Dohan et al., in which PRF membrane induced proliferation of HGFs and these effects were significantly higher on days 3, 7, 41 and 12 than the control group (26). The different results may be due to variations between individuals in the number of platelets, the interval between centrifugation of blood samples to obtain fibrin membrane and cultivation in the presence of the culture medium. It has also been observed that the proliferative effects of PRP on cells do not necessarily improve with increasing platelet concentration, but there is an optimal platelet concentration range. As shown in previous studies, an optimal platelet concentration is 2.5 times more than the normal blood for cells (27). However, platelet counts in the PRF cannot be determined due to platelet entrapment into the dense fibrin network (28).

PRF is a non-homogenous biomaterial, in which cells are cultured. This type of plasma contains different cytokines, leukocytes, plasma and proteins that are trapped in a highly dense



fibrin membrane. The fibrin matrix exerts significant effects on osteoblast differentiation. Also, the growth factors or PRF fibrin matrix structure may be a reason for dual behaviors in osteoblasts. In addition, PRF can lead to a controlled release of growth factors over time (28); as growth factors, such as TGF-B1 and PDGF-AB increased until day 41 and then decreased. PRF not only is able to release growth factors for a long time, but it can also delay the maximum release rates. Autologous growth factors have also had direct effects on cells for five to seven days (26).

In order to evaluate the total platelet product, it is necessary to use large culture plates to place the complete clot or platelet membrane without compressing the cells in the culture medium. A distance of at least 60 mm has been suggested for a possible interaction between platelet cells and cytokines and the development of cell proliferation (26, 29). In Tuan et al. study towards the role of fibrin in tissue healing showed that fibroblasts are able to regenerate the fibrin matrix and initiate the collagen producing (30).

There is still no consensus on the effects of leukocytes on platelet products. Some studies point to the potential of leukocytes in proliferation, differentiation and immunity, while the harmful biological effects of matrix metalloproteinase 8 and 9 present in neutrophils have been well established (11, 31). In addition, neutrophils can damage tissue by releasing large volumes of reactive oxygen types in the inflammatory phase (2). In future studies, it seems that the use of leukocyte-free platelet-derived products will be more cautious. In some studies, PRP was maintained at minus 80 degrees Celsius, which did not comply with clinical conditions (i.e, room temperature), which may change platelet and PRP properties (31). According to the previous studies, there are significant changes in growth factor concentrations (VFGF, PDGF, TGF- β , bFGF) between different individuals with similar platelet concentrations (2).

In the present study, all blood samples were obtained from a healthy volunteer to avoid any confounding factors. In this study, Emdogain® was used at two concentrations of fifty-one hundred $\mu\text{g} / \text{ml}$ with %2 FBS to evaluate the effect of Emdogain® on human gingival fibroblast cell proliferation. The Emdogain® study had significant proliferative effects at 24 and 72 hours. This increase in cell proliferation increased with increasing concentration of substance. According to Zeldich et al. Emdogain® in the absence of FBS showed no significant effect on fibroblast proliferation (17). In low concentrations of FBS (%0.2 -%2), Emdogain® proliferative effect emerged, so in the present study, Emdogain® was used in the presence of 2% FBS. In a recent study, Zeldich et al. used a 50 ml/ μg concentration to

investigate the effect of dose on the cell proliferation results. The present study was performed in two concentrations 50 ml/ μg and 100 ml/ μg with more depth. In a recent study in by Talebi et al the effect of Emdogain® and PRF on cell proliferation was performed and the results showed that both substances were equally effective in fibroblast proliferation (32).

The results showed that Emdogain® had a significant effect on cell proliferation compared with PRF at 72 h. However, this research was an in vitro study, according to which the results should be interpreted with caution and the obtained results cannot be fully generalized to clinical conditions. One of the limitations of this study was the relatively small number of samples due to the blood supply needed by only one volunteer. It is therefore recommended that future studies be carried out on the effect of these substances on other cell lines and primary cells using other methods of amplification analysis. In addition, evaluating the mechanism of action of these two substances could provide researchers with more details.

Conclusion

Under the study limitations, the most proliferation and viability of fibroblast cells were obtained after exposure to Emdogain® and the differences between Emdogain® and PRF were significant.

Conflict of Interest: 'None declared'.

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