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Original Article

Emdogain effect on gingival fibroblast adhesion in bioabsorbable and non-resorbable barrier membranes: An *in vitro* study

Mehrdad Barekatain, Morvarid Mafi[1], Shirin Amini[2], Shirin Zahra Farhad[2]

Department of Restorative Dentistry, Khorasgan Islamic Azad Dental School, [1]Department of Periodontics, Jundishapur University of Medical Sciences, Ahvaz, [2]Department of Periodontics, Khorasgan Islamic Azad Dental School, Isfahan, Iran

ABSTRACT

Background: Tissue engineering represents very exciting advances in regenerative medicine; however, periodontal literature only contains few reports. Emdogain (EMD) consists of functional molecules that have shown many advantages in regenerative treatments. This study investigated EMD effect on gingival fibroblast adhesion to different membranes.

Materials and Methods: Two dense polytetrafluoroethylene membranes (GBR-200, TXT-200), Alloderm and a collagenous membrane (RTM Collagen) were used in this experimental study. Each membrane was cut into four pieces and placed at the bottom of a well in a 48-well plate. 10 μg/mL of EMD was added to two wells of each group. Two wells were left EMD free. Gingival fibroblasts were seeded to all the wells. Cell adhesion was evaluated by means of a Field Emission Scanning Electron Microscope after 24 hours incubation. Data was analyzed by independent t-test, one-way and two-way ANOVA and post hoc LSD test. $P < 0.05$ in independent t-test analysis and $P < 0.001$ in one-way ANOVA, two-way ANOVA and *post hoc* LSD analysis was considered statistically significant.

Results: Alloderm had the highest cell adhesion capacity in EMD+ group and the difference was statistically significant ($P < 0.001$). In EMD- group, cell adhesion to TXT-200 and Alloderm was significantly higher than GBR-200 and collagenous membrane ($P < 0.001$).

Conclusion: This study showed that EMD may decrease the cell adhesion efficacy of GBR-200, TXT-200 and collagenous membrane but it can promote this efficacy in Alloderm. It also showed the composition of biomaterials, their surface textures and internal structures can play an important role in their cell adhesion efficacy.

Key Words: Alloderm, cell adhesion, emdogain, guided tissue regeneration, polytetrafluroethylene

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Address for correspondence:
Dr. Morvarid Mafi
Department of Periodontics, Jundishapur University of Medical Sciences, Ahvaz, Iran.
E-mail: Morvarid.ltk@gmail.com

INTRODUCTION

The main goal of periodontal treatment is to control the inflammation in periodontal tissues and to regenerate the lost tissues predictably. To meet this goal it is critical to guide the tissues capable of regeneration.[1-3] Guided tissue regeneration is an accepted method for enhancement of lost periodontal tissue. In this technique a barrier membrane is used to prevent epithelial cell migration and stabilization of the clot into the defect. This prevention results in the migration of periodontal ligament cells and osteoblasts into defect and these cells are known to be responsible for tissue regeneration.[4] Different types of barrier membranes are introduced that had shown favorable results due to different studies.[5] These membranes are different in composition and structure, but all of them prevent the migration of epithelial and gingival connective tissue cells into the defect and ideally, a barrier membrane should enhance the cell attachment and migration of the progenitor cells.[5-10] Wound healing is a complex process which includes

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cell migration, cell attachment to various extracellular matrix components, and cell proliferation.[11,12] Cell attachment process is a four-step sequence which includes adsorption of glycoproteins to the substrate surface, cell contact, attachment, and spreading.[9,10] Cell proliferation begins after these events.[5] Tissue integration property ensures the stabilization of the wound and inhibits the migration of epithelial cells, which results in better gain of clinical attachment levels.[13-15]

According to their degradation characteristics, barrier membranes are divided into two groups of resorbable and non-resorbable membranes. Collagen is the most common material used as resorbable membranes.[5] It facilitates hemostasis and wound stability by promotion of platelet aggregation along with fibroblast migration which accelerates wound closure,[16,17] but collagenous membranes are not stiff enough to resist soft tissue pressure during healing.[16,18]

Polytetrafluroethylene (PTFE) is the main composition of non-resorbable membranes.[19] Although their biocompatibility and positive effect on bone regeneration was shown, but a second surgery is required for their removal which may traumatize the newly formed immature periodontal tissue and causes patient discomfort and increases the treatment time and cost.[20] Also, the membrane stiffness may result in tissue dehiscence which is the main reason of treatment failure 3 weeks after membrane placement and exposes the membrane which leads to bacterial infection and decrease in the levels of gained clinical attachment.[21-24] An alternative to an expanded PTFE membrane is a high-density polytetrafluroethylene (d-PTFE) membrane which is commercially available as TXT-200 and GBR-200. High-density polytetrafluroethylene membranes have small porosities, so bacterial contamination is eliminated and therefore there is no need of primary closure when they are being used and they can be left exposed to the oral cavity.[25-28]

The acellular dermal matrix (Alloderm) was originally introduced in medicine for reconstructive plastic surgeries but is also used in dentistry in various periodontal procedures like root coverage and keratinized tissue augmentation around teeth and implants.[29-31] It has many advantages, but the absence of cells and vessels makes tissue incorporation slower, therefore, attempts of culturing fibroblasts on Alloderm were performed to achieve early wound healing and decrease wound contraction in periodontium.[32-35]

Fibroblasts play an important role in the healing process. It has been shown that the key factor in the success of regenerative treatment is the recruitment or delivery of cells to the defect site and the production of suitable extracellular matrix along with the periodontal tissues.[36,37]

Introduction of specific cell adhesion molecules to the membrane surfaces may lead to specific tissue responses. Different growth factors and proteins have been introduced and one of them is enamel matrix derivatives. A commercially available product of enamel matrix derivatives is called Emdogain® (EMD). It is an acidic extract of low molecular weight procine enamel proteins mainly amelogenin and a propylene glycol alginate vehicle.[38,39] Different studies showed that EMD enhances the adhesion, proliferation, and matrix production of periodontal ligament fibroblasts, stimulates cell growth, and production of insulin growth factor-1 and transforming growth factor-β1 in periodontal ligament cells although it has no appreciable effect on osteoblastic differentiation and has no effect on epithelial cells.[37,38] All of the described characteristics of EMD make it a suitable functional material for regenerative treatments. Therefore, its effects on cell adhesion to different materials were investigated in the present study.

There was also no available study that had compared the fibroblast adhesion among TXT-200, GBR-200, Alloderm, and collagenous membrane (RTM Collagen, Cytoplast®) or the effect of EMD on fibroblast attachment to these common barrier membranes. The present study was performed to compare cell adhesion among the prementioned membranes and also to investigate the effect of EMD on gingival fibroblast attachment.

MATERIALS AND METHODS

For this experimental *in vitro* study, gingival fibroblast cells (NCBI Codece C165) were provided by Pasteur Institute of Iran. Cells were cultured in a culture flask and cultured in the presence of Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) containing 10% Fetal Calf Serum and 100 μg/ml of penicillin, streptomycin, and amphotericin B. The flask was kept in 37ºC in a 5% CO_2 atmosphere in an incubator with humidity. The medium was

changed twice a week. Cells were cultured for 3 weeks and passaged for five times.

Four different barrier membranes were used in this study. Two non-resorbable dense polytetrafluoroethylene membranes GBR-200 (GBR1224, LOT: 2541) (Cytoplast®, Osteogenic Biomedical, Lubbock, TX, USA), TXT-200 (TXT1224, LOT: 3688) (Cytoplast®), RTM Collagen (RTM2030, LOT:C2030263) (Cytoplast®) and acellular dermal matrix (ADM, 302111, LOT: B42234) (Alloderm, Biohorizons, Birmingham, AL, USA).

Each membrane was cut into two 6×6-mm pieces and washed with sterile saline solution according to the supplier's instructions. In RTM Collagen and ADM groups, membranes were washed with sterile saline solution until the protect paper was floating. A 48 wells culture plate was used in this experiment. Five groups of four close wells were selected. Four groups were used for membranes (each group containing four wells for each membrane). All of the membranes were adapted at the bottom of the selected group of wells. No membrane was added to the fifth group and it served as a control group to check the growth of seeded cells. 10 µg/mL of EMD (LOT: C2822, Emdogain®, Straumann, Malmö, Sweden) was added to two wells of each group (EMD+) and two wells were left without any EMD (EMD-). Cells were seeded at a density of 100,000 cell/well on the membranes. Plate was placed in a 37ºC incubator with humidity and 5% CO_2 atmosphere for 24 hours. The growth of seeded cells in the fifth group was evaluated by means of a light microscope.

Then cells were washed four times with phosphate buffer saline (PBS) to remove non-adherent cells. The membranes were fixed in 2.5% glutaraldehyde for 2 hours, washed five times with distilled water for 20 minutes, treated with 1% osmium tetroxide for 1 hour, washed again five times with distilled water for 20 minutes and finally dehydrated through a series of graded ethanol solutions and left for 24 hours in room temperature to dry. To finish the process, they were coated with gold and analyzed with Field Emission Scanning Electron Microscope (Hittachi s4160, Stanford, CA, USA). An operator not aware of the experimental set up analyzed the membranes with SEM. Each membrane was divided into four intellectual parts under SEM with ×300 magnifications and one image was taken from each part. Another two observers totally unaware of the experiment counted the cells on each image and if there was a difference, the least cell count was recorded.

Data was analyzed by independent t-test, one-way ANOVA, two-way ANOVA, and *post hoc* LSD test with SPSS18 (version 18;SPSS Inc, Chicago, IL, USA). $P < 0.05$ in independent t-test analysis and $P < 0.001$ in one-way ANOVA, two-way ANOVA, and *post hoc* LSD analysis was considered statistically significant.

RESULTS

Figures 1-4 illustrates the membranes in EMD- and EMD+ groups under SEM with ×300 magnifications and Table 1 shows the gained data after cell counting process by two observes.

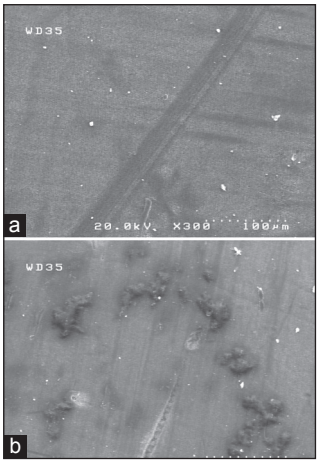

Figure 1: SEM illustration of GBR-200 membrane, a- EMD- group, b- EMD+ group

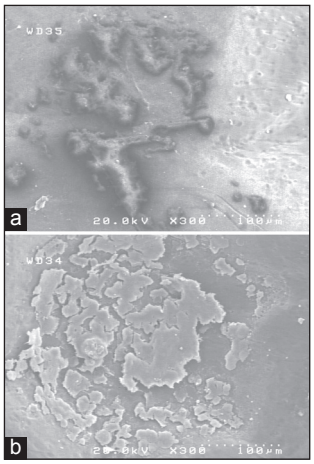

Figure 2: SEM illustration of TXT-200 membrane, a- EMD- group, b- EMD+ group

Figure 5 shows the mean of attached gingival fibroblasts to the barrier membranes used in this study in EMD+ and EMD- groups.

Two-way ANOVA test showed the membrane type ($P < 0.001$) and the presence of Emdogain ($P = 0.04$) affect the gingival fibroblast adhesion efficacy.

The quality of cell adhesion to each membrane in EMD+ and EMD- groups was evaluated by independent t-test and it was shown that cell adhesion in GBR-200 was slightly higher in EMD- group, but this difference was not statistically significant ($P = 0.060$). On the other hand, cell adhesion to TXT-200 membrane was higher in EMD- group and the difference was statistically significant ($P = 0.020$). Cell adhesion to RTM Collagen showed no significant difference between EMD+ and EMD- groups ($P = 0.310$). Unlike other membranes, ADM showed higher cell adhesion efficacy in EMD+ group and the difference was statistically significant ($P = 0.004$). All of the above results are illustrated in Figure 6.

One-way ANOVA also showed that ADM has the highest cell adhesion capacity in EMD+ group and the difference was statistically significant ($P < 0.001$). It

Table 1: The mean of attached cells to membranes in EMD+ and EMD- groups

Membrane	EMD	Attached cells mean (SD)
GBR-200	+	3.37 (1.76)
	–	5.75 (2.76)
TXT-200	+	8.62 (4.50)
	–	61.50 (57.80)
RTM Collagen	+	5.37 (2.32)
	–	4.12 (2.41)
ADM	+	56.23 (11.87)
	–	40.25 (6.08)

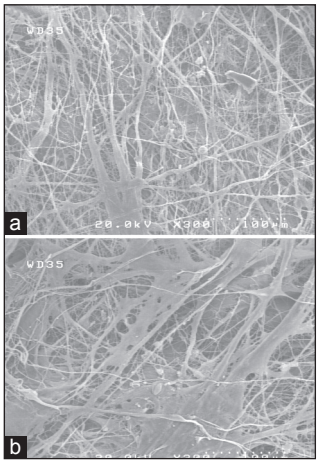

Figure 3: SEM illustration of RTM Collagen membrane, a- EMD- group, b- EMD+ group

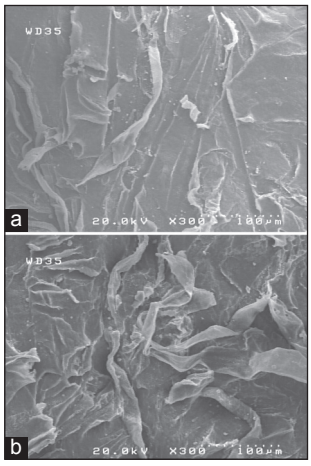

Figure 4: SEM illustration of ADM, a- EMD- group, b- EMD+ group

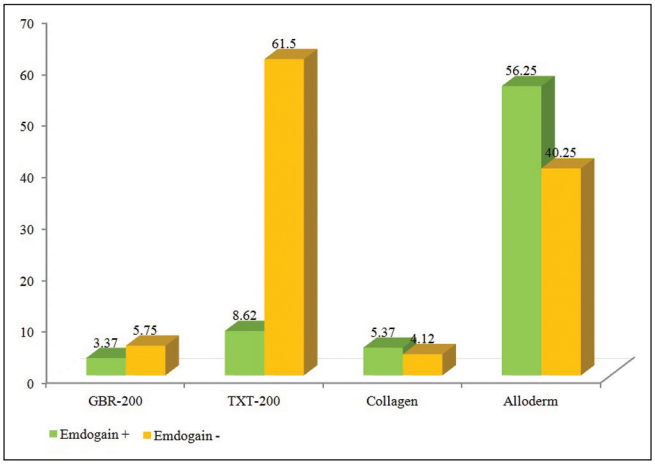

Figure 5: Mean of attached cells to membranes in EMD+ and EMD- groups

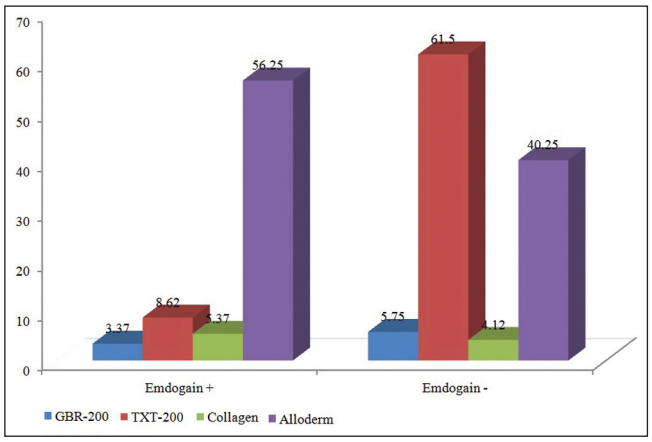

Figure 6: Mean of attached cells in EMD+ and EMD- groups to the studied membranes

was also shown that in EMD- group gingival fibroblasts adhesion to TXT-200 and ADM is statistically significantly higher in comparison to GBR-200 and RTM Collagen ($P < 0.001$).

Post hoc LSD test was used to compare membranes two by two. As it is shown in Figure 3, this test revealed when EMD is present, cell adhesion to ADM is higher than GBR-200 ($P < 0.001$), TXT-200 ($P < 0.001$), and RTM Collagen ($P < 0.001$). This test also showed when EMD is not present, cells significantly adhere to TXT-200 more than RTM Collagen ($P < 0.001$) and GBR-200 ($P < 0.001$). Also when EMD was not present, cell adhesion to TXT-200 was slightly higher than ADM, but it was not statistically significant ($P = 0.156$).

DISCUSSION

Tissue engineering represents very exciting advances in regenerative medicine; however, periodontal literature only contains few reports.[40-44] ADM has been shown as an useful material in gingival augmentation.[37] It has many advantages, but the absence of cells and vessels makes tissue incorporation slower.[36] In an attempt to solve this problem, fibroblasts were cultured on Alloderm as an alternative to achieve early wound healing and decrease wound contraction in periodontium.[32-35] In this study, EMD was used to enhance the gingival fibroblast adhesion to different membranes including Alloderm.

The highest cell efficacy in all of the studied groups belonged to TXT-200 in absence of EMD followed by ADM in the presence of EMD and then ADM in the absence of EMD. When EMD was not present, GBR-200 had slightly higher cell adhesion in comparison to the presence of EMD, but this difference was not significant ($P = 0.060$). Same happened to TXT-200, but the difference was significant ($P = 0.02$). Cell adhesion to RTM Collagen was slightly higher when EMD was present but the difference was not significant in comparison to the absence of EMD ($P = 0.310$).

The difference in the cell adhesion efficacy when EMD is present can be related to its mitogenic properties. Bertl *et al*.[45] observed that 0.1-50 μg/mL of EMD promotes cell migration in the wound healing process and it is inhibited at 100 μg/mL. Also, in other studies it was reported that the EMD with the concentration of 25 μg/mL and lower leads to better results,[46-48] so in the present study the concentration of EMD was considered 10 μg/mL for the EMD+ groups.

Hoang *et al*.[49] had shown that under physiologically relevant conditions, amelogenin (the main composition of EMD) does not bind to collagen. Van der Pauw *et al*.[48] declared that with collagen as a substratum, EMD has an inhibitory influence on periodontal ligament cells attachment and spreading. Lyngstadaas *et al*.[50] found a five-fold increase in cell adhesion on plates coated with EMD. These conflicting results may be due to the higher concentration of EMD (500 μg/mL) which was used by these authors.

In the present study, cell adhesion to RTM Collagenmembrane showed no significant difference in EMD+ and EMD- groups which was similar to some of the mentioned studies.[49,50] ADM which has a collagenous composition showed higher cell adhesion efficacy in the presence of EMD. This result was similar to Lyngstadaas *et al*.[50] study but the concentration of EMD which was used in the present study (10 μg/mL) was different form theirs (500 μg/mL). It can be concluded that ADM, *per se* has a good cell adhesion efficacy. It is derived from human skin and is prepared by a controlled process that removes epidermis and the cells from the dermis but leaves the basement membrane and extracellular matrix organization and collagen and elastin fibers undamaged.[29,32] Although RTM Collagen is a collagenous membrane, but similarity of ADM structure to human skin may be the reason of its better cell adhesion efficacy in comparison with RTM Collagen.

In EMD- groups, TXT-200 showed statistically higher cell adhesion in comparison to GBR-200 ($P < 0.001$) but in the presence of EMD this difference was not significant ($P = 0.118$). Although their composition is the same and they are both made of dense polytetrafluoroethylene, but their surface texture is different. TXT-200 has a roughened surface that is caused by the presence of macro-porosities on its surface but GBR-200 lacks these porosities [Figures 1 and 2]. It seems that EMD may cover the porosities of TXT-200and decrease the cell adhesion efficacy of this material.

These results show that surface texture and material structure play an important role on the cell adhesion efficacy. Cell adhesion affects the tissue integrity efficacy of biomaterials and higher tissue integrity efficacy results in better gain of clinical attachment levels.[13-15]

CONCLUSION

Within the limits of the present study, it is shown that the membranes used in this study affect cell adhesion, proliferation and differentiation of gingival fibroblasts. Also, EMD may lower the cell adhesion efficacy of GBR-200, TXT-200, and RTM Collagen but it can promote this efficacy in ADM. When membranes are used without EMD, TXT-200 shows the highest cell adhesion efficacy followed by ADM without a statistically significant difference.

This study also showed not only composition of biomaterials, but also their surface texture and internal structures may play an important role in their cell adhesion efficacy.

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