

Evaluation of the Effects of Enamel Matrix Protein Derivatives on Clinical Attachment Gain in Periodontal Defects and on Proliferation and Differentiation of Periodontal Ligament Fibroblasts *In Vitro*: A Double-blind Study

Mine Matriks Protein Türevlerinin Periodontal Defektlerde Klinik Ataşman Kazancı ve İn Vitro Ortamda Periodontal Ligament Fibroblastlarının Çoğalması ve Farklılaşması Üzerine Olan Etkilerinin Değerlendirilmesi: Bir Çift Kör Çalışma

© Ahu Dikilitaş¹, © Mehmet Taşpınar², © Bülend İnanç³

¹Uşak University Faculty of Dentistry, Department of Periodontology, Uşak, Turkey

²Aksaray University Faculty of Medicine, Department of Medical Biology, Aksaray, Turkey

³Trakya University Faculty of Dentistry, Department of Periodontology, Edirne, Turkey



Keywords

Enamel matrix derivatives, fibroblasts, guided tissue regeneration, periodontal ligament

Anahtar Kelimeler

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Address for Correspondence/Yazışma Adresi:

Ahu Dikilitaş MD,
Uşak University Faculty of Dentistry,
Department of Periodontology, Uşak, Turkey
Phone : +90 506 356 95 00
E-mail : ahuparlakdikilitas@hotmail.com

ORCID ID: orcid.org/0000-0003-4130-2526

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Abstract

Objective: This work assesses the effect of enamel matrix protein derivatives (EMD) on the periodontal ligament (PDL) fibroblasts *in vitro*, and its possible contribution to guided tissue regeneration (GTR) in the therapy of intrabony defects.

Materials and Methods: Forty-one intrabony defects were cured randomly with either EMD or GTR (22) or only with GTR (19). Osteogenic differentiation of PDL fibroblasts was measured using enzyme-linked immunosorbent method.

Results: EMD have been demonstrate to increase the proliferation rate, osteogenic differentiation and mineralization of PDL fibroblasts *in vitro*.

Conclusion: EMD has inductive impacts on PDL fibroblasts proliferation and differentiation *in vitro*, although it's possible contribution to periodontal attachment gain remains below a statistically significant level of clinical detection when combined with GTR.

Öz

Amaç: Bu çalışmanın amacı, mine matriks protein (MMP) türevlerinin *in vitro* ortamda periodontal ligament (PDL) fibroblastları üzerindeki etkisini ve kemik içi defektlerin tedavisinde yönlendirilmiş doku rejenerasyonuna (YDR) olası katkısını değerlendirmektir.

Gereç ve Yöntemler: Kırk bir kemik içi defekt rastgele MMP + YDR (22) ya da sadece YDR (19) ile tedavi edilmiştir. PDL fibroblastlarının osteojenik farklılaşması enzime-bağlı immünosorbent yöntemi ile değerlendirilmiştir.

Bulgular: MMP'nin *in vitro* ortamda PDL fibroblastlarının canlılığını, proliferasyon oranını, osteojenik farklılaşmasını ve mineralizasyonunu artırdığı bulunmuştur.

Sonuç: MMP'nin *in vitro* ortamda PDL fibroblastlarının çoğalması ve farklılaşması üzerinde endüktif etkileri vardır, ancak klinikte YDR ile birleştirildiğinde periodontal ataşman kazanımına olası katkısı istatistiksel olarak klinik tespit seviyesinin altında kalmıştır.

Introduction

Periodontal diseases are chronic inflammatory conditions described by destruction of dental supporting tissues (1). Periodontal regeneration includes the regeneration of cementum damaged by inflammation, regeneration of bone loss and primarily, the attachment of connective tissue fibers to the exposed root surface to re-form the connective tissue attachment (2). Biological mediators such as hard and soft tissue grafts, enamel matrix protein derivatives (EMD) and barrier membranes in guided tissue regeneration (GTR) have been used to provide regeneration (3,4).

GTR is defined as “preventing the migration of gingival epithelium and connective tissue cells to the defect area by placing a barrier membrane and allowing the periodontal ligament (PDL) and alveolar bone cells to migrate into the defect area” (5). It has been reported that GTR is a more successful method than open flap debridement in treating intrabony and furcation defects (6).

EMD are produced by the Hertwig’s epithelial root sheath along tooth development and when used in periodontal regenerative treatment, they enable the differentiation of mesenchymal cells originating from the tooth follicle into cementoblasts, and the formation of cell-free cementum (7). Various studies demonstrate that EMD can be used alone, with GTR, or in combination with bone grafts in curing periodontal intrabony defects, thus yielding different success rates (8-10).

Although the utilize of EMD together with many regenerative materials has been investigated in clinical or *in vitro* studies, our research explores its effects in clinical and *in vitro* setting combination. This work aims to evaluate the influence of EMD, in addition to GTR in intrabony defects in individual and the proliferation and differentiation of PDL fibroblasts *in vitro*.

Materials and Methods

The subjects included in this research were selected among individual who applicant to Yüzüncü Yıl University Faculty of Dentistry, Department of Periodontology, and diagnosed with chronic periodontitis following clinical and radiographic examination. This work was confirmed by the Yüzüncü Yıl University Faculty of Medicine Non-Drug

Clinical Research Ethics Committee (decision no: 05, date: 05.12.2013) and a written signed consent was obtained from all subjects.

Clinical Studies

Of the 41 defects in 33 patients, 19 were treated with EMD and GTR and 22 were treated using GTR alone.

Individuals who do not have any systemic disease, have not taken periodontal treatment in the last 6 months, do not use drugs affecting the periodontium, do not smoke, and have three-walled intrabony defect with a depth measurement higher than or equal to 3 mm were included.

Patients selected from one of each 2 treatment groups were randomly selected among patients who meting the inclusion criteria.

All periodontal assessments [Plaque index (PI), gingival index (GI), gingival bleeding index (GBI), gingival recession (GR), clinical attachment level (CAL) and periodontal probing depth (PPD)] were measured with a periodontal probe (PQW7 Williams, Hu-Friedy, Chicago, USA) and by a proficient calibrated periodontist (A.D.) who was unaware of the groups (11-13). Defect size (DS) was calculated with the Imaje J computer program at baseline and at 6 months.

Following data collection, surgeries was performed by a same periodontist (B.I.) not involved with clinical measurement. The study was double-blind.

Attachment loss (AL) and PPD were conducted in five patients with periodontitis who were not involved in this work for a calibration study. The intra-examiner credibility of the parameters was obtained by providing intra-class correlation coefficients of 0.88 for PPD and 0.84 for AL.

In the EMD and GTR group; after the root surfaces were dried, Prefgel™ (Straumann, Basel, Switzerland) was done for 2 min to remove the smear layer and operation area washed with serum. After the area was thoroughly dried, Emdogain® gel (Straumann, Basel, Switzerland) was done to root surfaces and defect area. And then, the collagen membrane (Evolution Membrane, Giaveno, Italy), prepared in accordance with the DS, was placed to completely cover the area (Figure 1). No probing or subgingival debridement was performed during the 6-month follow-up period.

In Vitro Studies

Preparation of Primary Cell Culture from Human PDL Fibroblasts Cells

PDL fibroblasts tissue was taken from healthy premolar teeth extracted for orthodontic purposes



Figure 1. Treatment of intrabony defect in the EMD + GTR group. A: Before the operation, B: After the flap elevation, C: Intrabony defect area, D: Emdogain gel applied to root surfaces and intrabony defect covered by collagen membrane E: The surgical site after the suture, F: 6 months after surgical treatment, G: Periapical radiography at baseline, H: Periapical radiography at 6 months after EMD: Enamel matrix protein derivatives, GTR: Guided tissue regeneration

from individuals between the ages of 18-26 (14). The PDL fibroblasts tissue was cut into small parts and added in a cell culture flask including a solution of Dulbecco's Modified Eagle's Medium [(DMEM)-including L-glutamine and glucose-enriched formulation], 10% fetal calf serum (FCS, Sigma), 1% penicillin and streptomycin (pen/strep) and 1% non-

essential amino acid, and then transferred to an incubator providing an atmosphere of 95% humidity and 5% CO₂ at 37 °C.

Preparation of Experimental EMD Solution

The experimental enamel matrix solution was prepared using Emdogain®, a commercially available

periodontal regeneration product. Emdogain concentration is 30 mg/mL. To achieve the proper concentration, 100 mL of this enamel matrix solution was mixed with 9.9 mL of DMEM with 10% FBS to form a final 10 mL of enamel matrix solution of 100 mg/mL. The experimental solutions used (25, 50, 100 and 200 mg/mL) were obtained by diluting 100 mg/mL enamel matrix solution with 10% FBS with the appropriate amount of DMEM.

PDL Fibroblasts Cell Passages

PDL fibroblasts culture time is ~4-6 weeks until PDL fibroblasts proliferates around explant tissues. Following the first passage, cell count was made using a hemocytometer.

Cell Viability and Proliferation Assay

Four different concentrations of EMD solution were added to wells for four days; at the end of each day, 10 µL of the MTT cell viability kit [3-(4,5-Dimethylthiazolyl)-2,5-Diphenyltetrazolium Bromide] solution with a concentration of 5 mg/mL was added to the well and placed in an incubator for 4 h. Absorbance wavelength at which the results were read was set at 590 nm, and the reference wavelength was read at 620 nm.

Preparation of Osteogenic Induction Experimental Group

For the purpose of creating osteogenic induction experimental groups, dexamethasone with a concentration of 10⁻⁶ M, ascorbic acid with a concentration of 0.005 g/mL, and β-glycerophosphate with a concentration 216.04 g/mol were added and mixed. To form EMD experimental groups at four different concentrations, 0.005, 0.01, 0.02, and 0.04 mL of Emdogain® were added, respectively.

Evaluation of Mineralization After Differentiation (Alizarin Red)

PDL fibroblasts cells obtained from the third passage were placed in 24-well culture dishes after centrifugation and resuspension. Mineralization foci associated with *in vitro* osteogenesis were viewed under an inverted light microscope with Alizarin staining.

Identification of Osteogenic Markers After Differentiation-osteocalcin and Bone Sialoprotein

The phrase of bone sialoprotein and osteocalcin markers, which are osteogenesis markers, were examined using enzyme-linked immunosorbent assay

(ELISA) to show osteogenic differentiation at the cellular level. In our study Human Bone Sialoprotein ELISA Kit and Human Bone Osteocalcin ELISA Kit, were used.

Statistical Analysis

SPSS 15 (SPSS Inc., Chicago, IL, USA) statistical package software was utilized for analyzing data. The Kolmogorov-Smirnov normality test was applied to understand whether the data were normally distributed Wilcoxon test and Kruskal-Wallis test were used. Spearman's rho correlation test was utilized to examine the amount and the direction of change in variables. In evaluating the statistical significance of results, a 95% confidence interval and a 0.05 significance level were considered.

Results

A sum of 33 individuals, 21 females and 12 males (mean age = 33.29 ± 6.77) were recruited this study. The clinical parameters of the EMD + GTR and GTR groups were compared. Statistically significant difference was found among PI, GI, GBI parameters only after treatment ($p < 0.05$) (Table 1).

In a four-day assessment of the cell numbers different EMD concentrations, on the fourth day, a statistically significant difference was reported among the number of cells obtained from the three teeth at the 25 µg/mL EMD concentration ($p < 0.05$). In the cell proliferation percentage assessment over four days in different EMD concentrations and three different teeth, only the difference between the percentage of cell proliferation obtained from the three teeth at the 50 µg/mL EMD concentration on the first day and the 25 µg/mL EMD on the fourth day was reported to be statistically significant ($p < 0.05$) (Table 2).

After differentiation, foci of mineralization associated with *in vitro* osteogenesis were found to be 20% at 3 weeks and 25% at 4 weeks in wells containing the osteogenic induction + EMD experimental group (Figure 2).

The difference between bone sialoprotein levels obtained from three different teeth at two different concentrations was not statistically significant ($p > 0.05$), but the difference among osteocalcin levels was statistically significant ($p < 0.05$) (Table 2).

Table 1. Distribution of the number of patients, number of defects, age and gender by the groups and comparison of clinical parameters of EMD + GTR and GTR groups after treatment

		EMD + GTR	GTR	p
Demographic variables	Number of patients	18	15	
	Number of defects	22	19	
	Mean age	33.04±6.16	33.57±7.58	
	Gender	Male	4	
		Female	11	
Clinical parameters (Mean ± SD) (After treatment)	PI	0.26±0.12 [#]	0.19±0.09 [#]	0.04*
	GI	0.33±0.08 [#]	0.24±0.11 [#]	0.012*
	GBI	0.38±0.06 [#]	0.30±0.05 [#]	0.0001***
	PPD (mm)	3.09±0.62	3.04±0.68 [#]	0.51
	GR (mm)	2.71±0.73 [#]	2.65±0.58 [#]	0.143
	CAL (mm)	5.55±1.04 [#]	5.22±0.84 [#]	0.180
	DS (mm ²)	2.11±0.83 [#]	2.28±0.82 [#]	0.639

[#]After treatment is statistically significantly different from before treatment (p<0.05), *p<0.05. ***p<0.001 (statistically significant) EMD: Enamel matrix protein derivatives, GTR: Guided tissue regeneration, SD: Standard deviation, PI: Plak index, GI: Gingival index, GBI: Gingival bleeding index, PPD: Periodontal probing depth, GR: Gingival recession, CAL: Clinical attachment level, DS: Defect size

Table 2. Distribution of the cell numbers, proliferation, bone sialoprotein and osteocalcin percentage in two different EMD concentrations

	Days	EMD	Tooth I	Tooth II	Tooth III	p
Cell numbers (Mean ± SD)	I	25 µg/mL	8456.6±582.0	6067.0±2530.3	7878.6±624.0	0.202
		50 µg/mL	8489.0±598.0	8087.3±983.2	8204.3±495.4	0.051
	II	25 µg/mL	18257.3±1893.1	18101.6±5502.4	232777.0±3423.9	0.561
		50 µg/mL	6257.3±1314.5	8319.3±1077.9	7314.3±3106.2	0.733
	III	25 µg/mL	9644.6±1556.7	9853.3±791.1	11199.3±1269.3	0.561
		50 µg/mL	10576.6±1218.3	10696.3±1527.7	10492.3±1533.9	0.875
	IV	25 µg/mL	8292.66±193.49	7748.33±122.68	10644.33±993.33	0.032*
		50 µg/mL	9585.66±814.94	8974.33±447.70	10861.00±1063.64	0.288
Proliferation percentage (Mean ± SD)	I	25 µg/mL	31.66±6.48	5.33±2.96	27.00±3.78	0.061
		50 µg/mL	24.66±3.84	2.66±1.33	28.66±5.81	0.050*
	II	25 µg/mL	160.33±27.06	158.00±78.42	232.00±48.77	0.561
		50 µg/mL	5.33±4.81	25.00±11.53	44.00±23.35	0.211
	III	25 µg/mL	44.00±16.19	40.33±11.28	59.66±18.18	0.561
		50 µg/mL	50.33±17.37	52.00±21.77	49.33±21.95	0.875
	IV	25 µg/mL	18.00±2.88	10.00±1.73	51.66±14.07	0.032*
		50 µg/mL	36.66±11.68	27.66±6.56	54.66±15.07	0.288
Bone Sialoprotein (ng/mL)		25 µg/mL	28.11±16.51	36.02±4.31	39.16±9.11	0.668
		50 µg/mL	33.09±8.81	37.29±5.26	37.69±8.10	0.794
Osteocalcin (ng/mL)		25 µg/mL	29.83±7.65	31.92±5.62	47.66±9.44	0.025*
		50 µg/mL	27.77±0.61	31.89±8.20	50.46±20.43	0.026*

EMD: Enamel matrix protein derivatives, SD: Standard deviation, *p<0.05

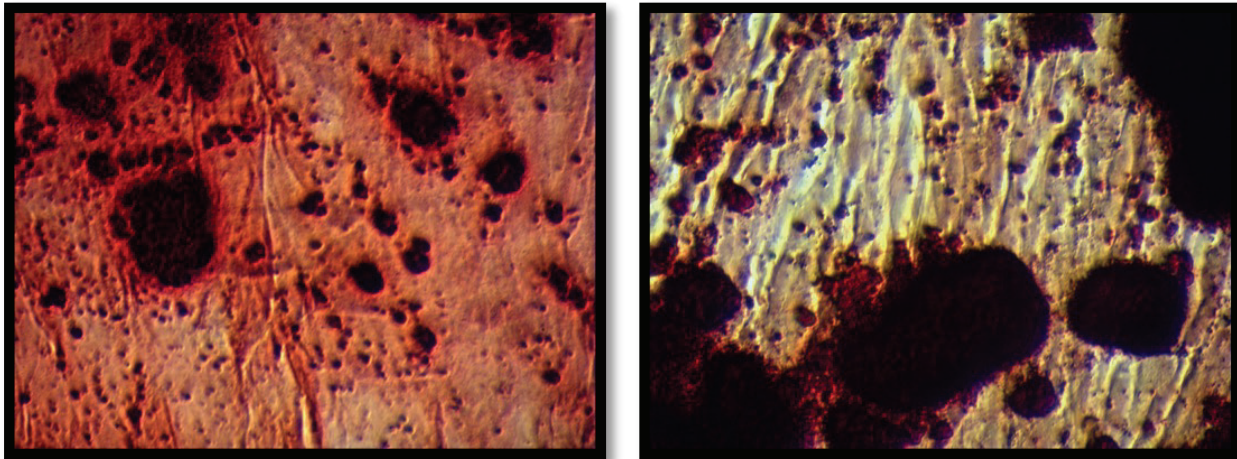


Figure 2. Appearance of wells containing osteogenic induction and EMD experimental group in invert light microscope after Alizarin red staining (3 weeks-4 weeks)

Discussion

Periodontal regeneration refers to the complete restoration of the periodontium in terms of structure and function. To provide periodontal regeneration, several biological mediators such as hard and soft tissue grafts, barrier membranes and EMD are used (3,4,8). This study purposes to determine the efficacy of EMD in chronic periodontitis subjects with intrabony defects and to investigate its effects on PDL cells *in vitro*.

When the clinical indexes were compared, PI, GI and GBI values significantly decreased after treatment compared to baseline, and the decline was higher in the group where only GTR was applied. The results showed that PI, GI and GBI parameters are related to each other and can prevent inflammation and bleeding with effective plaque control methods. When the clinical parameters of PPD, CAL and GR amount in EMD + GTR and GTR groups are compared, both groups showed a decrease in PPD and CAL and an increase in GR amount; however, there was no significant difference between them. In a study by Simonelli et al. (15), it was reported that EMD does not ensure any supplemental advantage in terms of clinical parameters. According to the results of our study, EMD application does not ensure any supplemental clinical advantage in regenerative treatment. However, there are differences between studies investigating the effect of using EMD in combination with other regenerative treatments in bony defects (16-18). This may be due to the choice

of patients and types of defects do vary between different studies. Because the flap is not stable in defects where EMD is applied, it is necessary to use regenerative materials such as barrier membrane and bone graft that can support EMD in the defect area.

To examine the biological effect of EMD at the cellular level, the viability and proliferation rate of PDL fibroblasts were examined. Rodrigues et al. (19) and Cattaneo et al. (20) demonstrated that EMD enhancement the proliferation of PDL fibroblasts (19,20). In our study, we observed that EMP increases the viability and proliferation of PDL fibroblasts cells. Wang et al. (21) reported that enamel matrix proteins caused to thicken the PDL cell layers and these reinforced cell layers have good mineralization ability in terms of osteogenic differentiation (21). Kato et al. (22) reported that enamel matrix proteins increased the proliferation of PDL fibroblasts cells and increase alkaline phosphatase activity, boost the production of osteonectin and osteocalcin stimulating calcified nodule formation and increased mineralization. In an *in vitro* study, it was observed that EMD increases osteogenic differentiation by inducing mineralized nodule formation and Ca deposition (23). The data from our study is compatible with other studies in showing that EMD increases the levels of bone sialoprotein and osteocalcin, which are osteogenic markers at the cellular level.

Rodrigues et al. (19) demonstrated that EMD increased mineralization foci associated with osteogenesis at the end of a three-week period. Kato

et al. (22) reported that EMD derivative amelogenin protein caused calcified foci in PDL fibroblast cells and increased odontoblastic differentiation. Consistent with other studies, the results of our study show that EMD increases the mineralized focus formation at the cellular level.

Conclusion

The conclusion of this study confirms the hypothesis that EMD is a fine indication for clinical use, particularly when targeting hard tissue gain; however, it may not provide measurable attachment gain at the clinical level.

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Ethics

Ethics Committee Approval: This work was confirmed by the Yüzüncü Yıl University Faculty of Medicine Non-Drug Clinical Research Ethics Committee (decision no: 05, date: 05.12.2013).

Informed Consent: Written signed consent was obtained from all subjects.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: A.D., B.İ., Concept: A.D., B.İ., Design: A.D., M.T., B.İ., Data Collection or Processing: A.D., M.T., B.İ., Analysis or Interpretation: A.D., M.T., B.İ., Literature Search: A.D., M.T., Writing: A.D.

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