Effects of Dentin Extracts on Human Periodontal Ligament Cells *In Vitro*

Dentin Ekstraktlarının İnsan Periodontal Ligament Hücreleri Üzerine *İn Vitro* Etkileri

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ABSTRACT Objective: Dentin extracts (DE) has been shown to have the potential to alter cell functions within the periodontal microenvironment. The aim of this study is to determine the effects of DEs on human periodontal ligament (PDL) cells *in vitro*. **Material and Methods:** Human PDL cells were obtained from healthy premolars. DE was prepared by sequential extraction procedures where 4M guanidine followed by 4M guanidine/0.5M EDTA was used. Proliferation of cells, total protein amount and mineralized nodule formation were recorded. **Results:** Significant increase in proliferation by time was observed in all groups except DE+Dexamethasone (Dex). Only significant difference was noted between DE and Dex+DE at day 11. Treatment of PDL cells with Dex, DE or Dex+DE decreased total protein amount compared with the control group at day 5. At day 7, statistical difference was recorded between Dex and Dex+DE. At day 11 there was statistical difference between DE and Dex+DE. Mineral-like nodules were observed in all groups containing mineralization media and increase in the size and quantity of mineral-like nodules were observed in cultures containing DE. **Conclusion:** DE had an enhancing effect on proliferation and mineralized nodule formation of PDL cells. It can be concluded that DE may show promising effects on differentiation of PDL cells which can be interpreted as it may be useful for regenerative procedures. Studies performed with different extraction procedures on different cells types composing of the periodontium are needed to clarify the exact role and mechanism of the possible action of DE in periodontal regeneration.

Key Words: Dentin; periodontal ligament; regeneration; *in vitro*

ÖZET Amaç: Dentin ekstraktlarının periodontal mikro çevrede hücre fonksiyonlarını etkileyebilme özelliğine sahip oldukları gösterilmiştir. Bu çalışmanın amacı dentin ekstraktlarının insan periodontal ligaman (PDL) hücreleri üzerine etkilerinin *in vitro* olarak değerlendirilmesidir. **Gereç ve Yöntemler:** Çalışmada premolar dişlerden elde edilen sağlıklı insan PDL hücreleri kullanıldı. Dentin ekstraktları (DE) çekilmiş insan dişlerinden, 4M guanidini takiben 4M guanidin/0.5M EDTA kullanılarak gerçekleştirilen ekstraksiyon aşamaları ile elde edildi. Dentin ekstraktlarının hücre proliferasyonu, total protein miktarı ve mineralize nodül oluşturma kapasitesi değerlendirildi. **Bulgular:** DE+Deksametazon (Dex) grubu dışındaki tüm gruplarda zamana bağlı olarak proliferasyon oranında belirgin artış saptandı. İstatistiksel olarak anlamlı olan tek fark 11. günde DE ve Dex+DE grubu için saptandı. PDL hücrelerine Dex, DE veya Dex+DE uygulanmasının kontrol grubuna oranla 5. günde total protein miktarında azalmaya neden olduğu gözlendi. Yedinci günde, Dex ve Dex+DE arasında, 11.günde ise DE ve Dex+DE grupları arasında istatistiksel farklılık tespit edildi. Mineralizasyon ortamı içeren tüm gruplarda mineralize nodül formasyonu gözlenirken, DE içeren kültürlerde mineralize nodül miktarı ve boyutunda artış tespit edildi. **Sonuç:** Çalışmamızda DE'nın, PDL hücrelerinin proliferasyonu ve mineralize nodül formasyonu üzerine olumlu etkileri gözlenmiştir. Çalışmanın sonuçlarına dayanarak, DE'nın PDL hücrelerinin farklılaşması üzerine umut verici etkileri olabileceği ve rejeneratif işlemlerde fayda sağlayabileceği öne sürülebilir. DE'nın periodontal rejenerasyondaki kesin rolü ve etki mekanizmasının saptanabilmesi için periodonsiyumu oluşturan farklı hücre tipleri üzerinde, farklı ekstraksiyon prosedürleri ile elde edilen DE kullanılarak yapılacak olan ileri çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Dentin; peryodontal bağ; rejenerasyon; canlı dışında

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Periodontitis, the most common inflammatory disease causing tooth loss in adults, is characterized with progressive destruction of tooth supporting apparatus including gingiva, alveolar bone, periodontal ligament and cementum.[1,2] The ultimate goal for treatment of periodontal disease is the complete regeneration of the lost structures by the coronal reestablishment of the periodontal ligament (PDL) together with corresponding cementum and supporting alveolar bone.[3] Regeneration of these periodontal tissues can be achieved by appropriate stimulation of cells within the periodontal ligament. The required agents for stimulating these cells can be derived from the surrounding areas such as proteins from alveolar bone, cementum and dentin.[4]

Dentin has been considered to have the potential to alter cell function within the periodontal microenvironment.[5] It has been suggested that the organic component of demineralized dentin and cementum might stimulate cell activities, including early cell migration, attachment, orientation and fibroblast adherence for PDL cells which can also be predictable for periodontal regeneration.[6,7]

PDL represents a soft tissue structure possessing a neurovascular supply, extracellular matrix composed mainly of type I collagen and noncollagenous proteins as well as multiple cell types such as fibroblasts, macrophages and polymorpholeukocytes.[8] Convincing evidence exists supporting the concept that periodontal cells, when triggered appropriately, have the capacity to synthesize fibrous connective tissue, cementum and bone.[9] *In vitro* biochemical data indicate that the periodontal ligament contains a heterogeneous cell population, with some cells exhibiting more osteoblastic properties. These properties can be summarized as; high alkaline phosphatase (ALP) levels, a PTH-mediated cyclic AMP response (cAMP), ability to form mineralized-like nodule, *in vitro* and increased synthesis of bone 'gla' protein in response to 1a,25 $(OH)_2$ vitamin D_3.[10]

Dexamethasone (Dex) is a synthetic glucocorticoid that has catabolic affects on bone while administered systemically. Dex has been shown to selectively enhance osteoprogenitor cell proliferation and to promote differentiation of osteoprogenitor cells into osteoblastic cells *in vitro*.[11] Combined applications of Dex with beta glycerophosphate and ascorbic acid has been reported to increase mineralized nodule formation in periodontal ligament cells and rat bone marrow stromal cells.[12,13]

Based on the knowledge that PDL cells play a major role in promoting periodontal regeneration and they have the capability of producing mineral-like nodules *in vitro*, this study was designed to determine the effects of dentin extracts on proliferation and protein synthesis of human periodontal ligament cells *in vitro*. Also, effects of dentin extracts on mineralization of human periodontal ligament cells were evaluated.

MATERIAL AND METHODS

CELL ISOLATION AND CULTURE

Human PDL cells were obtained from healthy premolars (without any signs of periapical/periodontal infection) extracted from adolescent patients (ages between 12-14) due to orthodontic reasons.[10,13,14] Written informed consent forms were obtained from patients before providing the samples. Immediately after extraction, teeth were placed in biopsy media consisting of Dulbecco's Modified Eagle's medium (DMEM) (Seromad Biochrom KG Berlin, Sigma Chemical Co., St.Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Seromad Biochrom KG Berlin, Sigma Chemical Co., St.Louis, MO), 250 mg/mL gentamicin sulfate, 5 mg/mL amphotericin B, 100 U/mL penicillin, and 100 mg/mL streptomycin. The mid-third portion of the periodontal ligament was collected carefully by scraping with a surgical scalpel, and minced into small pieces. After rinsing with biopsy medium, PDL pieces were plated in culture flasks containing biopsy media, and incubated overnight at 37 ^{0}C in an atmosphere supplemented with 5% CO_2 and 95% air. The media was changed with culture medium (DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin) and maintained with complete change of media every other day until the PDL cells became confluent.

Confluent PDL cells (day 14) were passaged with 0.005% trypsin-0.02% EDTA and cells between the sixth and eighth passage were used for all experiments.

PREPARATION OF DENTIN EXTRACTS

Dentin extracts were provided from extracted teeth, without any decay, endodontic treatment or periapical infection by using the extraction procedures that have been previously described.[15,16] Briefly; periodontal ligament and cementum were removed mechanically with sharp scalers until dentin was exposed all over the surface. Exposed dentin was washed with cold distilled water. A sequential extraction procedure was used where 4M guanidine, followed by 4M guanidine/0.5M EDTA separates tissue matrices into mineral-non associated and mineral-associated soluble protein fractions, respectively. The mineral associated fraction (guanidine EDTA extracts of dentin) is thought to be more likely to contain the proteins involved in cell matrix interactions and therefore used in our experiments. These extracts were first filtered through Whatman no-1 filters and then concentrated by ultra filtration under pressure through Amicon YM-10 filters. Concentrated extracts were dialyzed against distilled water, lyophilized and stored at -20 ^{0}C until use.

PROLIFERATION AND PROTEIN SYNTHESIS ASSAYS

PDL cells were plated at a density of $3x10^4$ cells/cm^2 in 24 well-plates in DMEM containing 10% FBS. After 24 hours of incubation, the medium was changed to DMEM containing the appropriate treatment agents according to the study design. The experimental study groups were designed as:

Group I (Control): 10% FBS

Group II (Dex); 10% FBS+Dexamethasone (Dex) (10^{-7}M),

Group III (DE): 10% FBS+Dentin Extracts (50 mg/ml)

Group IV (Dex+DE): 10% FBS+DE+Dex (10^{-7}M)

All experiments were performed in triplicate. Media was changed every 2 days and proliferation of cells was determined on days 1, 7 and 11 with Neubauer hemacytometer. Total protein amount was measured by using the Lowry method[17] on days 1, 5, 7 and 11.

MINERALIZATION ASSAY

PDL cells were cultured in $3x10^4$ cells/cm^2 in 35 mm dishes in DMEM containing 10% FBS in triplicate. After 24 hours, the medium was changed to DMEM containing the appropriate treatment agents according to the study design. The experimental study groups were designed as:

Group I (Control): 10% FBS (Control)

Group II (Dex): 10% FBS and mineralization media (50 mg/ml ascorbic acid, 10mM b-glycerophosphate)+ dexamethasone (Dex, 10^{-7}M)

Group III (DE): 10% FBS, mineralization media and DE (50 mg/ml)

Group IV (Dex+DE); 10% FBS, mineralization media, Dex (10^{-7}M) and DE (50 mg/ml). Media was changed every 3 days and throughout the 30 day observation period, cells were stained weekly to evaluate mineral-like nodule formation using the von Kossa method.[18] All experiments were performed in triplicate.

The results were analyzed with t- test by using the Instat 2.1 biostatistics program (GraphPad Software, Inc., San Diego, CA).

RESULTS

PROLIFERATION AND PROTEIN SYNTHESIS ASSAYS

PDL cell proliferation assays were performed at days 1, 7 and 11. Significant increases by time were observed in all groups except Group 4 (Dex+DE) in which cell number slightly decreased at day 11 (Figure 1). When groups were compared, no statistical differences were reported between the groups at day 1 and 7. Only statistically significant difference was noted between Group 3 (DE) and Group 4 (Dex+DE) at day 11 ($p<0.05$) (Table 1).

Protein synthesis analyses were evaluated at days 1, 5, 7 and 11. Data for protein synthesis analysis are presented in Figure 2 and Table 2. Treatment of PDL cells with Dex alone ($p<0.01$), DE alone ($p<0.05$) or Dex+DE ($p<0.01$) statistically decreased the total protein amount when compared with the control group at day 5. At day 7, statistical difference was only recorded between Group 2 (Dex) and Group 4 (Dex+DE) ($p<0.05$). At day 11, significant

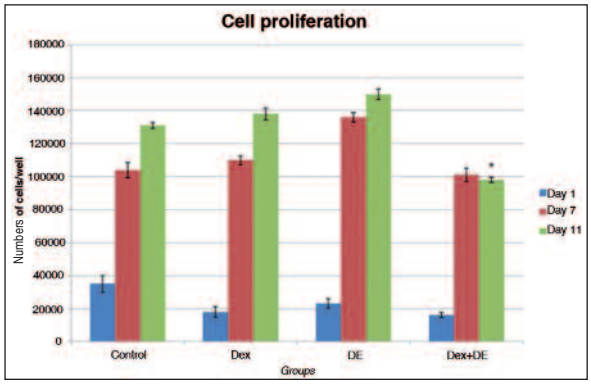

FIGURE 1: Proliferation assay data. Increased cell number by time was observed in all groups except Dex+DE Group at day 11.

Dex: Dexamethasone; DE: Dentin extracts.

* p<0.05 compared to DE.

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TABLE 1: Proliferation assay data. (Numbers of cells/well) (All experiments were performed in triplicate).

Groups	Day 1	Day 7	Day 11
Control	$3.5x10^4$	$10.4x10^4$	$13.1x10^4$
Dex	$1.8x10^4$	$11x10^4$	$13.8x10^4$
DE	$2.3x10^4$	$13.6x10^4$	$15x10^4$
Dex+DE	$1.6x10^4$	$10.1x10^4$	9.8x104*

Dex: Dexamethasone; DE: Dentin extracts.

*p<0.05 compared to DE.

difference was noted between Group 3 (DE treatment alone) and Group 4 (Dex+DE) (p<0.01).

MINERALIZATION ASSAY

Von Kossa staining of the cells were shown in Figure 3. Mineral-like nodules were observed in all groups containing mineralization media beginning on days 16 and 20 (Figure 3b, c and d). Increase in the size and quantity of mineral-like nodules were observed in cultures containing dentin extracts (Figure 3d). Addition of DE increased the staining for mineral-like nodules. The most prominent staining for mineral-like nodules was noted in the group treated with Dex+DE (Figure 3d). The formation of mineral-like nodules in size and extent were similar in Dex and DE groups.

DISCUSSION

Dentin is formed by deposition of hydroxyapatite on to the organic matrix composed of proteoglycans, glycoproteins, sialoproteins, phosphoproteins and other molecules.[19-21] The structural components of dentin matrix have been of great importance for researchers for several years due to its potential role in the regenerative process. It has been shown that besides composing of many non-collagenous proteins, dentin contains various growth factors such as; insulin-like growth factor (IGF I, II), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), placenta growth factor (PlGF), fibroblast growth factor (FGF) and transforming growth factor (TGF).[22-26] Bone morphogenetic protein (BMP), known as an osteoinductive factor, has also been isolated from dentin and bone.[27]

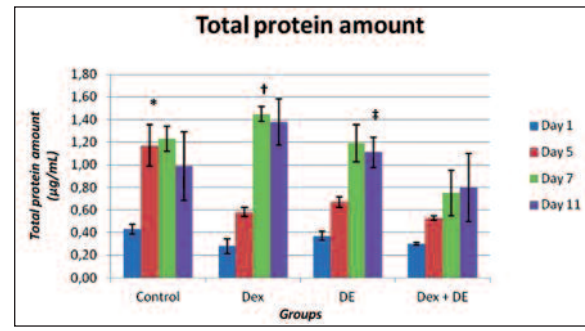

FIGURE 2: Total protein assay data. Decreased protein amount was reported in all groups at day 5 when compared to the control group.

Dex: Dexamethasone; DE: Dentin extracts.

* p<0.05 compared to all groups at day 5.

† p<0.05 compared to Dex+DE.

‡ p<0.01 compared to Dex+DE.

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TABLE 2: Total protein assay data (µg/mL) (All experiments were performed in triplicate).

Groups	Day 1	Day 5	Day 7	Day 11
Control	0.43±0.04	1.17±0.18*	1.23±0.11	0.99±0.30
Dex	0.28±0.06	0.58±0.04	1.45±0.06†	1.38±0.20
DE	0.37±0.04	0.67±0.04	1.19±0.16	1.11±0.13‡
Dex + DE	0.3±0.01	0.53±0.02	0.75±0.20	0.80±0.30

Dex: Dexamethasone; DE: Dentin extracts.

* p<0.05 compared to all groups at day 5.

† p<0.05 compared to Dex+DE.

‡ p<0.01 compared to Dex+DE.

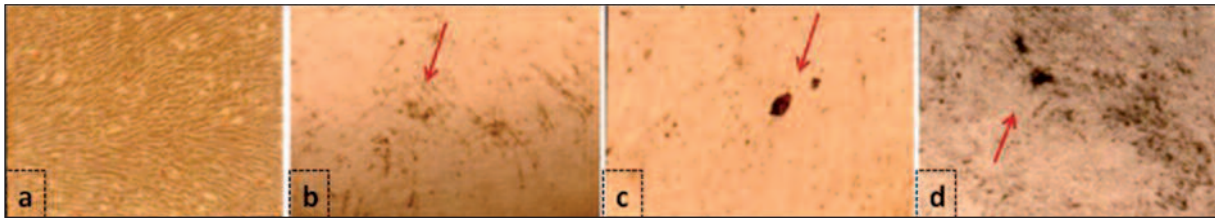

FIGURE 3: Mineralization experiments **(a, b, c and d).** Mineral-like nodules were observed in all experimental groups beginning on days 16 and 20. In groups containing DE, increase in size and quantity of mineral-like nodules can be noted.
a: Control; **b:** Dexamethasone (Dex); **c:** Dentine extracts (DE); **d:** Dex+DE.
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It has been shown that demineralized dentin induces osteogenesis and chondrogenesis *in vivo* when implanted at ectopic sites, induces chondrogenesis of muscle derived fibroblast and cartilage formation by embryonic muscle derived mesenchymal cells *in vitro*.[28-32] Enhancement of osteogenesis has been reported following implantation of decalcified dentin into oral sites including extraction sockets, drill holes and bony defects.[31,33,34]

The regenerative properties of DE can be attributed to the growth and differentiation factors present in the matrix of dentin. Thus, the remaining organic component of dentin following extraction procedures is an important issue to be concerned. Although several methods are reported in the literature for extraction of dentin, to what extent each procedural step damages or inactivates the regenerative component still remains unknown.

Based on the results of our study, significant increase in proliferation by time was observed in all groups except Dex+DE. Only significant difference noted for proliferation was between DE and Dex+DE at day 11. Treatment of PDL cells with Dex, DE or Dex+DE decreased total protein amount compared with the control group at day 5. At day 7, statistical difference was recorded between Dex and Dex+DE. At day 11 there was statistical difference between DE and Dex+DE. Mineral-like nodules were observed in all groups containing mineralization media and increase in the size and quantity of mineral-like nodules were observed in cultures containing DE.

In the present study we used the method previously described by Somerman et al. for dentin extraction.[35] In this *in vitro* study using the same extraction procedure performed on gingival fibroblasts, slightly increased proliferation rate for dentin extracts and similar total protein productions for bone and dentin were reported.[35] In another study, Takata et al. investigated the effects of DE on MC3T3-E1 cells. The results indicated that exposure of cells to dentin changed the morphology of cells into a more fibroblastic cell-like appearance and suppressed proliferation. Northern-blot analysis performed in that study revealed that dentin extracts inhibited osteoblast differentiation and decreased mRNA expression for bone sialoprotein (BSP) and Osteocalcin (OCN).[36] Although using the same extraction procedures, different types of cells used in these studies constitute a challenge in comparing our results directly. To the best of our knowledge, there's only one study in the literature evaluating the effects of DE on PDL cells. In this study Devecioglu et al. compared the effects of freeze dried dentin demineralized with 0.6 N HCl, on PDL cells and osteoblasts with coralline hydroxyapatite, cementum and cryopreserved bone. Similar to our study, the results reported higher proliferation of PDL cells for dentine and coral hydroxyapatite. Additionally higher mineralized nodule formation was observed in dentine treated osteoblasts, indicating the potential effect of dentin on differentiation.[6]

The findings of our study demonstrated that guanidine EDTA extracts of dentin had suppressed proliferation on day 1 in all groups, in consistent with the study of Takata et al.- in which guanidine

EDTA extracts of dentin were shown to suppress cell proliferation in a dose-dependent manner in osteoblastic cells.[36] Following the suppression of proliferation on day 1, enhanced PDL cell proliferation was observed in all groups at all time periods examined. However, addition of Dex to DE decreased cell proliferation at day 11, which can be explained by the shift from proliferation to differentiation of cells present in the culture. It has been reported in the literature that addition of Dex to non-mineralizing media turned the morphology of PDL cells into a more spindle shape form when compared to cultures without Dex, suggesting the potential role of Dex on PDL cell differentiation.[13] Conversely, in the present study, it has been shown that while addition of Dex alone and DE alone increased proliferation of cells, combination of Dex and DE suppressed proliferation at day 11 which may be attributed to the possible synergistic effects of DE and Dex on differentiation. The effects of Dex on osteoblast cells are in accordance with the present study that, when applied in mineralizing media, Dex has been reported to enhance proliferation.[37]

It has been shown that PDL cells form mineral-like nodules formation when supplemented with mineralization media. Consistently, the findings of the present study revealed mineral-like nodule formation of PDL cells for all groups treated with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate and addition of DE improved size and quantity of mineral-like nodules formed. These results confirm that PDL cells can be induced to form mineral-like nodules *in vitro* and demonstrate that dentine extracts can increase the formation of mineral-like nodules in PDL cells which may be interpreted as the enhancing effect of DE on differentiation of PDL cells.[38] In a recent study, Chandrasekaran et al. demonstrated that exogenous treatment with dentin matrix protein 1 (DMP-1) activated Alkaline phosphatase gene expression and mineral like nodule formation in PDL cells-indicating osteoblast differentiation.[39]

Recently, DE have also been used in clinical applications based on the results of *in vitro* studies showing promising effects on regeneration of different cell types. Clinical application of demineralized dentin matrix with different barrier membranes in regenerative procedures performed in bone defects in rabbits showed accelerated bone healing and larger quantity of bone tissue formation for dentin extracts applied with different barrier membranes.[40-42] Although differing from our study in methodology for extraction, in which extraction was performed with 0,6 N HCl , the results of these clinical studies support the findings of the present study by showing promising regenerative effects also in clinical applications.

Based on the promising results observed both in *in vitro* and *in vivo* studies, DE has gained increasing importance in tissue engineering applications. In a recent study, Yang et al. used treated dentin matrix (TDM) as a biological scaffold with embedded dental follicle cells (DFCs).[43] The results of the study showed that implantation of DFCs combined with TDM to the dorsum of mice revealed higher expression of extracellular matrix proteins such as Collagen 1 (COL1), integrin 1β, fibronectin, ALP, bone sialoprotein and osteocalcin. Based on the results of these results, authors suggested that DFCs combined with TDM may prove an appropriate construction of tooth root and could be an alternative approach for treatment of root/tooth defect of loss in the future.

Results of the present study indicated that DE had an enhancing effect on proliferation and mineral-like nodule formation of PDL cells. Based on the results of our study, it can be concluded that DE show promising effects on differentiation of PDL cells which can be interpreted as it can be useful for clinical regenerative procedures. For achieving complete periodontal regeneration, the exact role of DE on different cell types composing the periodontium (alveolar bone, gingiva and cementum) needs to be clarified. Potential regenerative effects of dentin extracts can be attributed to its organic components including several growth factors and bone morphogenetic proteins, which can be affected by the extraction method used. Thus, studies evaluating the components of dentin extracts subjected to different extraction procedures are needed to find the most effective extraction

method for regeneration. Further *in vitro* and clinical studies performed on different cell types of the periodontium and using different extraction procedures are needed to provide basis for more accurate and consistent clinical applications.

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