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ORIGINAL ARTICLE

Enhancing attachment of human gingival fibroblasts to periodontally compromised teeth: A comparative analysis of hyaluronic acid, EDTA, enamel matrix derivatives

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Ethylenediaminetetraacetic acid;
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Abstract *Background:* Gingival recession is a characteristic indicator of periodontitis and one of the factors that contributes to increased tooth clinical crown length. Patients with root exposure frequently undergo periodontal surgical procedures in addition to adjunctive therapy to increase root coverage area and soft tissue stability.

Purpose: This study aimed to evaluate fibroblast-root surface adhesion and determine whether periodontitis-damaged root surface microstructure can be restored using ethylenediaminetetraacetic acid (EDTA) and an enamel matrix derivative (EMD), individually or in combination.

Material and methods: Teeth extracted from patients with periodontal disease were used to create 60 samples, with each group containing six specimens. The test groups were provided root planing or root condition-specific materials (hyaluronic acid [HA], 24% EDTA, EMD, or EDTA/EMD) for varying treatment time periods. In contrast, the control group did not undergo any surface modifications. The samples and fibroblast cells were incubated for 72 h. The number of living cells on the root surface in each group was calculated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe nyltetrazolium bromide (MTT) assay (cell viability assessment).

Results: The control, root planing, and EMD groups showed that the root surfaces treated with EDTA for 4 min had significantly better cell adhesion. Surface EDTA treatment for 2 min significantly promoted cell attachment compared to root planing treatment. The root surfaces modified with EDTA/EMD for 2 and 4 min showed significantly improved cellular migration and adhesion compared to the root surface treated with root planing.

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Conclusion: EDTA and EDTA/EMD substantially affected the root surface, which was related to the length of the treatment process. This effect shifts the surface properties, alters fibroblast interactions with the root surface, and recruits more cells to cover a larger area.

1. Introduction

Periodontitis is a multifactorial inflammatory disease that causes gingival recession by affecting the periodontium, which comprises the supracrestal attachment, cementum, periodontal ligament (PDL), and alveolar bone (Papapanou et al., 2018; Slots, 2017). The primary etiology of periodontitis is bacterial biofilm formation (Lamont & Hajishengallis, 2015). The calculus is conducive to bacterial colonization adjacent to the structure surrounding the teeth (Beem et al., 1991). In the oral cavity, periodontal pathogenic bacteria produce toxins that destroy the periodontal tissue (Darveau et al., 2002) and trigger an inflammatory phase to eradicate bacterial invasion (Hajishengallis, 2015), ultimately resulting in alveolar bone resorption, cementum contamination, and destruction of the PDL. This deepens the pocket depth or promotes apical gingival margin migration, resulting in root surface exposure.

The root surfaces exposed to the oral cavity changed and differed from those not exposed to the oral cavity. Owing to cementum resorption, the root surface became rougher and contained bacterial endotoxins, and the cemental layer orifices were blocked by the bacterial biofilm (Bouchard et al., 1997; Moghare Abed et al., 2013; Torabinejad et al., 2003). These alterations prevent fibroblasts from adhering to the root surface and impair the ability of surgical periodontal therapy to cover the root surface and restore gingival attachment (Aleo et al., 1975; Pitaru et al., 1987). Fibroblasts produce gingival collagen fibers that connect the gingiva to the root surface (dentogingival fibers) and have a high turnover capacity to maintain soft tissue integrity around the teeth (Giannopoulou & Cimasoni, 1996). To cover the root surface using surgical techniques, the soft tissue stability around the teeth relies on the number of fibroblasts adhering to the surface. Surface features are fundamental for promoting fibroblast migration and attachment (Liu et al., 2020), and increasing collagen production to connect the soft tissue to the dental root.

Historically, scaling and root planing (SRP) therapy objectives have been used to eliminate subgingival calculus and alter the root surface by removing toxic cementum, creating a smoother surface texture, and removing the smear layer to improve cell adhesion (Aleo et al., 1975). However, SRP may not completely eliminate the smear layer (Rocha et al., 2015), which may affect the adhesion of fibroblasts. Several root conditioning materials, such as hyaluronic acid (HA), ethylenediaminetetraacetic acid (EDTA), citric acid, and tetracycline, have been proposed for root surface texture modification to improve surface compatibility and heighten fibroblast bonds with cementum or dentine (Jalaluddin et al., 2020; Mueller et al., 2017; Pitaru et al., 1984; Theodoro et al., 2010). These materials are applied to the root surface to demineralize the cementum or dentin layers, clear the cementum orifices from the smear layer, expose the dentinal tubules, and eradicate toxins to provide a healthy surface foundation for fibroblast migration and adhesion.

To the best of author knowledge, although Enamel Matrix Derivative (EMD) has been studied in vivo to evaluate the clinical outcomes of the application of EMD on roots combined with soft tissue periodontal surgeries, no study has yet evaluated the root surface alteration ability of EMD to increase fibroblast adhesion on the root surface.

1.1. Aim

This study aimed to evaluate the effects of EMD application on the periodontoloy involved root surface by measuring the number of viable fibroblasts that adhered to the damaged root.

2. Material and methods

2.1. Sample collection and preparation

Thirty periodontally involved teeth were collected from private dental clinics; all teeth were scaled to eliminate attached calculus without removing the cementum structure, and then autoclaved. Each tooth was decapitated using a new disc bur and sectioned into equally sized pieces, producing 60 samples (n = 60).

2.2. Fibroblast isolation

A periodontist at the dental teaching hospital (Umm Al Qura University) obtained soft tissue samples from patients selected for crown-lengthening surgery. The patient underwent a 6-week course of plaque control. Soft tissue features must be firm and resilient with no signs of inflammation or bleeding upon probing before surgery. All patients provided written informed consent. During the operation, keratinized gingival soft tissue was collected, preserved in saline solution, and immediately transported to the laboratory for fibroblast extraction.

Gingival tissues were rinsed with phosphate-buffered saline (PBS) and incubated in dispase 1 mg/mL (Sigma, USA) at 4 °C for 12 h. The epithelial layer was removed, the connective tissue was sliced into pieces, cultured in a 25 mL flask cell growth medium, and incubated at 37 °C in a humidified atmosphere with 5% CO_2. The complete growth medium for cell culture contained Dulbecco's Modified Eagle Medium (DMEM; Gibco Thermo Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Thermo Scientific, Logan UT, USA), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma, USA), and 2.5 µg/mL amphotericin B (Gibco).

2.3. Experimental design

Samples were divided into ten groups (each group had six samples): one untreated surface control group, one group treated with root planing (RP) (mechanical modification) only, and eight groups treated with different root conditioning chemical agents (HA, EDTA, EMD, and EDTA/EMD) for two different treat-

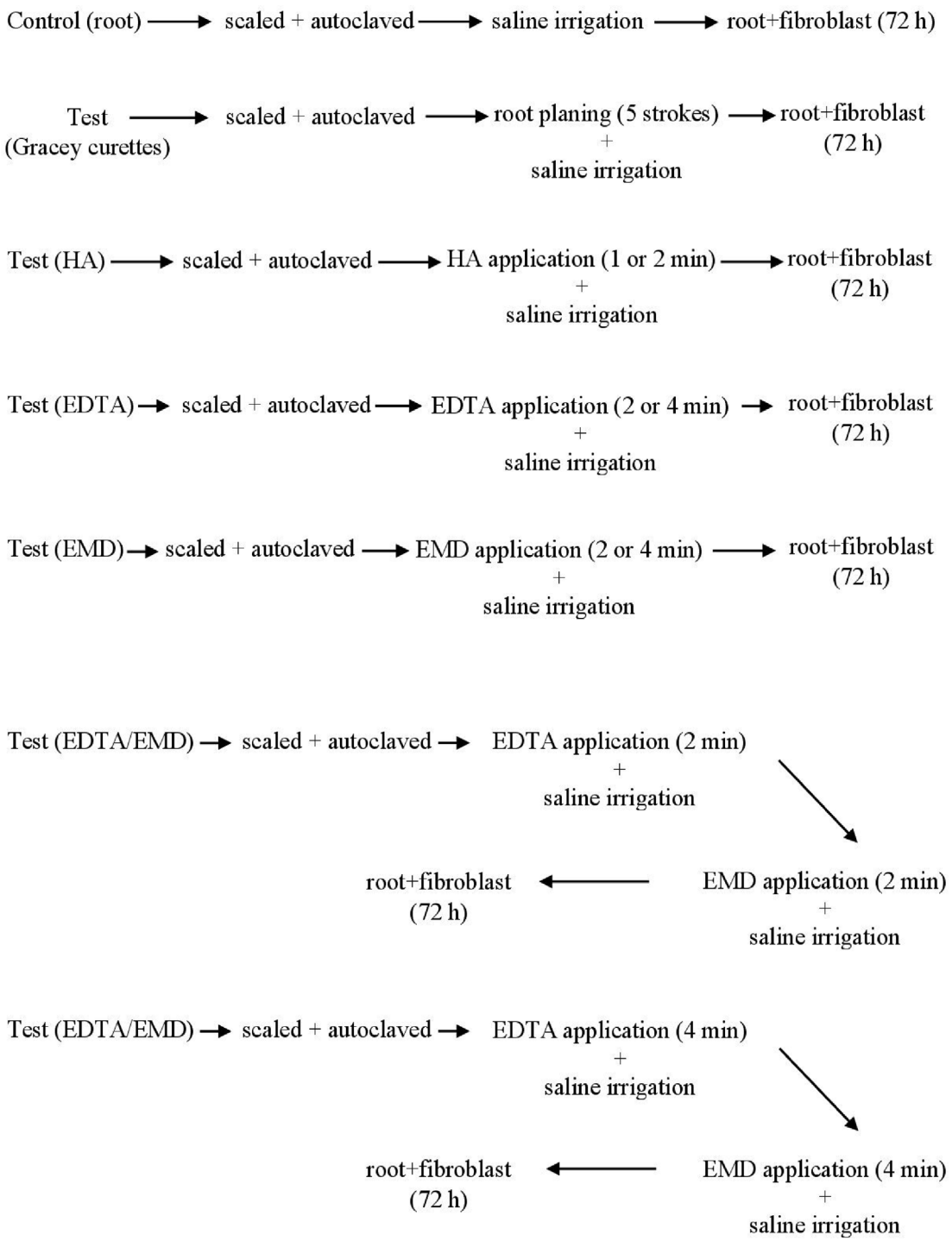

Fig. 1 Overview of Experiment Design.

ment time periods (short- and long-time application) without mechanical instrumentation (Fig. 1). All root samples were stored in saline containers for the experiment. The experiment was conducted at Umm Al Qura University's microbiology laboratory.

2.4. Root surface biomodification

The samples in the control group were washed with saline without mechanical or chemical surface modifications. One test group received mechanical instrumentation consisting of five Gracey curette strokes without chemical alterations. Six test groups were subjected to three different chemical treatments: two groups received HA Gel (Regedent AG, Zürich, Switzerland), two groups received 24% EDTA gel (Biodinâmica, Lisbon, Portugal), and two groups received EMD (Straumann, Basel, Sweden). For each chemical treatment, the material was applied for two different time periods (HA for 1 or 2 min and EDTA or EMD for 2 or 4 min) to two dif-

ferent samples. The final two test groups were subjected to EDTA/EMD. In one group, EDTA was applied to the root surface for 2 min, a saline rinse, then EMD application for 2 min. Another EDTA/EMD group used the same process; however, EDTA and EMD were applied for 4 min. A microbrush was used to rub the chemical agents over the root samples. Before cell culture for fibroblast adhesion to the root surface, all the specimens were rinsed five times with saline (10 mL) and stored for dehydration.

2.5. Cell culture

The primary gingival fibroblasts were planted on top of each root sample at a density of 2×10^4 cells/well in 500 μL complete growth medium. Plates containing root samples and fibroblasts were incubated for 72 h. After the incubation period, the root samples with attached fibroblasts were transferred to a 48-well plate for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (Talebi-Ardakani et al., 2017).

2.6. Cell viability and MTT assay

The MTT assay is a method used to measure cellular metabolism, indicating the total amount of living cells in a particular area**.** Fibroblasts adhered to root samples were incubated in DMEM (500 μL/well) containing 0.5 mg/mL MTT (Thermo Fisher Scientific, USA) in a 48-well plate for 3 h at 37 °C. The samples were separated from the medium following the incubation period, and a dimethyl sulfoxide (DMSO):isopropanol (1:1) solubilization solution was added to dissolve the formazan crystals for 30 min. The solution was then transferred to a 96-well plate at a concentration of 100 μL/well. The optical density (OD) of each well was measured at 570 nm using a spectrophotometric microplate reader (SpectroStar Nano; BMG Lab).

2.7. Statistical analysis

SigmaPlot version 14.0 software (Systat Sofware Inc., San Jose, CA, USA) was used for the data analysis. As the data were not normally distributed (Shapiro-Wilk), analysis of variance (ANOVA) on rank factor treatments (short and long) was used at different time points. This was followed by pairwise multiple comparison procedures using Tukey's post-hoc test ($P < 0.001$).

3. Results

In general, there was a statistically significant difference between the treatment groups ($P < 0.001$) (Kruskal-Wallis one-way ANOVA on ranks). A multiple comparison procedure (Tukey's post hoc test) was used to isolate the groups that differed from the others. Accordingly, surface treatment with EDTA for 2 min (shorter time period) enhanced fibroblast attachment to the surface of the specimen compared to curette surface treatment ($P < 0.05$). However, when EDTA was smeared on the surface for 4 min (longer time period), cell migration and connection to the surface increased compared to the cell-root attachment in the control, curettes, and EMD (longer time period) ($P < 0.05$). Cell movement and adhesion to the root surface modified with EDTA/EMD for 2 and 4 min (shorter and longer time periods, respectively) were greater than those of the RP-treated surface ($P < 0.05$). The surface alteration that occurred along the HA lining for 2 min (longer time period) encouraged cell connection more than the surface treated with curette-handed instruments ($P < 0.05$). The results are summarized in Table 1.

EDTA/EMD (2 min) expanded the fibroblast connection to the root surface, exhibiting the highest median in the short time period experiment, exceeding the number of cells connected to the untreated root sample or the surface modified with HA (1 min), EDTA (2 min), or EMD (2 min). However, the differences in median cell number among the groups specified earlier were not significant ($P = 1.000$). The number of cells attached to the root surface that were treated with EDTA (4 min) was higher than those treated with EDTA/EMD (4 min) or HA (2 min), with no significant differences ($P = 0.995$ and 0.855, respectively). EDTA/EMD (4 min) showed the second-highest fibroblast viability in the long-term experiment, followed by EDTA (4 min). The differences were not significant when the cell viability results from EDTA/EMD (4 min) were compared to those of the untreated surface or those treated with HA (2 min) or EMD (4 min) ($P = 0.203$, 0.999, and 0.116, respectively).

4. Discussion

One of the key functions of EDTA in soft tissue attachment is the elimination of toxic cementum, dentin, and the smear layer. Through its potent chelation process, EDTA removes calcium from the dentin layer, opens dentinal tubules (Deari et al., 2019), and provides an opportunity for gingival fibroblasts to insert collagen, thereby linking the soft tissue with the tooth structure. The smear layer on the root surface was efficiently and rapidly removed using EDTA (Rödig et al., 2010). The elimination of the smear layer, which comprises calculus, cementum particles, and bacteria that block the tubules and orifices, stimulates the formation of a connective tissue link in contrast to the epithelial attachment that may develop if the smear remains on the surface for an extended period (Polson & Proye, 1983). According to Mello et al. (2010), applying EDTA for prolonged time periods results in more significant outcomes. EDTA administered to the root samples for 4 min resulted in the highest level of cell volume attachment throughout the experiment. EDTA was able to generate a more biocompatible root surface and stimulate cell behavior for cell-root attachment to a greater extent than surfaces treated with conventional RP, which, in this study, was ineffective against periodontal disease-induced surface microstructure deterioration. Therefore, EDTA stimulates the development of a root surface that is more amenable to repair after surface microstructure degradation due to periodontal disease. Similar surface modifications were observed after 2 min of EDTA treatment, with particularly enhanced cell adhesion compared with the untreated and RP-treated surfaces. A higher number of cells adhered to the surface after treatment with EDTA for 4 min than after 2 min. The duration of EDTA treatment is critical for altering the rate of cell migration to the root surface.

Table 1 Median (IQR) MTT cell viability assay of fibroblast attachment after treatment with conditioning agents at two different time periods.

	Control (No TTT)	RP (curettes)	HA	EDTA	EMD	EDTA/EMD
Short time	0.199 (0.391)	0.031 (0.005)	0.471 (0.433)	0.391 (0.018)[††]	0.511 (0.826)	0.561 (0.5)[**]
Long time	0.199 (0.391)	0.031 (0.005)	0.471 (0.256)[@]	0.878 (0.505)[*†$]	0.149 (0.017)	0.669 (0.898)[#]

TTT = Treatment, IQR = Interquartile range (75–25 percentile).
[††] Significant difference between EDTA (Short time) and RP (short time period) (Tukey test; $P < 0.05$).
[@] Significant difference between HA (long time period) and RP (short time period) (Tukey's test; $P < 0.05$).
[*] Significant difference between EDTA (long time period) and RP (short time period) (Tukey's test; $P < 0.05$).
[†] Significant difference between EDTA (long time) and EMD (long time) (Tukey test; $P < 0.05$).
[**] Significant difference between EDTA + EMD (Short time) and RP (short time) (Tukey test; $P < 0.05$).
[$] Significant difference between EDTA (long time period) and No TTT (Tukey's test; $P < 0.05$).
[#] Significant difference between EDTA + EMD (long time period) and RP (short time period) (Tukey test; $P < 0.05$).

Clinical evidence supports the use of EMD to enhance root surface area coverage with soft tissue postsurgical therapy. In conjunction with soft tissue augmentation, EMD enhances the amount of gingiva adhering to the exposed root (Adriana-Castellanos et al., 2006; França-Grohmann et al., 2019; Pilloni et al., 2006). During tooth development, ameloblasts, which are highly specialized cells, secrete a protein known as the enamel matrix protein (EMP). EMD suppresses epithelial cell proliferation during periodontal regeneration (Kawase et al., 2000, 2002). This allows connective tissue cells to adhere to a greater proportion of the root surface. Adhesion between surface connective tissue cells is stronger and more reliable than that between surface epithelial cells. In addition to inhibiting epithelial cell growth, EMD increase connective tissue cell proliferation (Kawase et al., 2000, 2002). Over the past two decades, EMD has been researched and utilized as an additional therapy to recover exposed root surfaces. The greater the root surface area (Adriana-Castellanos et al., 2006), the more stable the treatment over time when using a root coverage surgical technique involving EMD (Spahr et al., 2005).

Although EMD has a significant impact on cell biology, the material was washed off the root surface prior to the application of fibroblasts; therefore, the experimental design did not permit an assessment of EMD at the cell level. Thus, this investigation focused on the capacity of EMD to affect the root surface. The absence of a statistically significant difference between the control and RP-treated groups regarding cell adhesion to the root surface treated with EMD suggests that it has a modest effect on cell adhesion owing to minor root surface alterations.

The combined EDTA/EMD treatment of the root surface enhanced cell attachment compared to EMD treatment alone. Although both the EDTA and EMD surface modifications enhanced cell adhesion (Akira, 2014), the specificity of the EDTA surface modification demonstrated that it was more favorable for gingival fibroblast attachment. This was confirmed by observing the effect of EDTA on cell adhesion. Because adding EDTA to the mixture (EDTA/EMD) for surface modification improves root surface soft tissue coverage and clinical outcomes (Górski & Szerszeń, 2022), EDTA therapy may improve therapeutic outcomes.

5. Conclusion

EDTA modified the root surface to promote fibroblast adhesion, and this effect was observed even when EDTA was applied before EMD. The duration of material on the root surface is an additional factor that can influence fibroblast adhesion. Longer application duration can lead to more favorable alterations on the surface that promote cell attachment.

CRediT authorship contribution statement

Baher Khaled Felemban: Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical approval

The study was approved by the local committee for biological and medical ethics in Umm Alqura University.

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