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ORIGINAL ARTICLE

Effect of root conditioning agents hyaluronic acid, EDTA and chlorhexidine on the attachment of human gingival fibroblasts to healthy root surface

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KEYWORDS

Gingival fibroblasts;
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Cell viability

Abstract *Background:* The gold standard treatment of periodontal diseases is scaling and root planing (SRP). Different adjunctive root conditioning agents such as hyaluronic acid (HA), ethylenediaminetetraacetic acid (EDTA), and chlorhexidine (CHX) have been used with SRP to improve the smear layer removal and the healing of periodontal tissues. The aim of this study was to compare the effect of manual scaling with or without HA, EDTA, or CHX root conditioning on the attachment and viability of human gingival fibroblasts (GF).

Methods: Fifteen healthy single rooted teeth were co llected and divided randomly into a scaled (n = 12) and non-scaled control group (n = 3). The scaled roots were subdivided equally into four groups; the first group did not receive any chemical treatment, while the remaining groups were treated with the conditioning agents HA or 17% EDTA or 0.2% CHX gel. Gingival fibroblasts were seeded on the top of each root and incubated for 48 h to allow attachment to the roots. The viability of fibroblasts attached to the root surface was assessed using MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay.

Results: The cell viability was the highest in the scaled only group ($p = 0.0001$) while the lowest was in the scaled with EDTA group ($p > 0.05$). The scaled group was the highest followed by the HA and CHX groups, while the EDTA group showed the lowest mean value.

Conclusion: SRP remains the superior method for regaining cell attachment to the root surface, leading to better periodontal health, and adjunctive therapies did not enhance the GF attachment to

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the root surface beyond the effect of SRP. Further studies are needed to investigate the effect of root conditioning agents on periodontally diseased teeth in vitro and compare them in vivo.

1. Introduction

The scaling and root planing (SRP) procedure is the gold standard for the non-surgical treatment of chronic periodontitis. However, SRP may be unsuccessful in removing the smear layer completely from the root surface. This may negatively affect the healing process by interfering with the reattachment of cells to the root surface. For this reason, different adjunctive therapies, such as chemical treatment, high energy laser treatment and the antimicrobial photodynamic therapy (Karam et al., 2017) have been used with SRP to improve the smear layer removal and consequently enhance healing (Cekici et al., 2013). The chemical treatment involves using different root conditioning agents such as ethylenediaminetetraacetic (EDTA), citric acid, phosphoric acid and tetracycline hydrochloride acid. These agents have been used to dissolve the smear layer and expose the collagen on the root surface, thus promoting the attachment of fibroblasts to the root during the healing process (Andrade et al., 2013; Theodoro et al., 2010). The effect of different root conditioning agents on viability and attachment of gingival fibroblasts to the root surface has been shown in multiple studies. EDTA (24%) has increased fibroblast attachment on the root surface (Babay, 2001). Tetracycline treatment after SRP was superior in removing the smear layer and tubule exposure in comparison with minocycline, doxycycline and citric acid (Chahal et al., 2014; Shetty et al., 2008). It has been shown that laser and root conditioning agents as an adjunctive to SRP have a comparable effect in removing the smear layer (Theodoro et al., 2010). Tetracycline HCl and EDTA removed the smear layer similarly, but EDTA has the advantage of producing larger dentinal tubules, which are better for cell attachment (Nanda et al., 2014). It has been found that citric acid, EDTA and maleic acid (MA) were all successful at removing the smear layer at one or three minute time intervals, while intergroup comparison indicated that MA had better smear layer removal ability than citric acid and EDTA (Ballal, 2017). Chlorhexidine (CHX) is one of the most commonly used oral antiseptics because it has a bactericidal effect on the pathogens of the periodontium (Giannelli et al., 2008). When CHX mouthwash was used as an adjunctive to SRP, there was a significant reduction in the plaque index and pocket depth after seven- and thirty-days follow-up (Alshehri et al., 2015). Recently, hyaluronic acid (HA) has been used as a root conditioning agent. HA is a natural component of the extracellular matrix that is present in different body fluids, organs and tissues, including the periodontium (Eliezer et al., 2019). The anti-inflammatory, antibacterial, anti-edematous and osteoinductive properties of HA made it an ideal material for enhancing periodontal wound healing (Bansal et al., 2010; Dahiya and Kamal, 2013). It has been shown that HA produces a rough dentin surface and significantly improves the attachment and spreading of cultured periodontal ligament cells on the dentin surface (Mueller et al., 2017). In a recent in vivo study, subgingival application of 0.8% hyaluronan gel in combination with SRP showed a statistically significant decrease in the pocket probing depths, and relative attachment level (Shah et al., 2016). The results of previous studies regarding choosing favorable root conditioning agents that enhance fibroblast attachment are still controversial. Thus, the aim of this study was to compare the effect of manual scaling with or without adjunctive root conditioning agents on the viability and attachment of cultured gingival fibroblasts (GF) to healthy root surfaces.

2. Materials and methods

This study was approved by Institutional Review Board (number 106-18) at the Faculty of Dentistry, Umm Al-Qura University.

2.1. Specimen collection and preparation

Fifteen healthy, sound, single-rooted teeth were collected from orthodontic clinics in Makkah post-extraction. The teeth were stored in normal saline for one week at room temperature. The crown of each tooth was cut using a tapered diamond bur. After that, the roots were divided randomly into scaled (n = 12) and non-scaled (n = 3) control groups. The scaled group received 20 apico-coronal moderate strokes per surface using Gracey curettes. The roots were then disinfected with 70% methanol and washed twice with phosphate-buffered saline (PBS) for two minutes (Fig. 1). Afterwards, the scaled roots were subdivided equally into four groups; the first group did not receive any chemical treatment, while the remaining three groups were treated with different conditioning agents: hydent BG: HA Gel composed of a mixture of cross-linked (1.6%) and natural (0.2%) Hyaluronic Acid (REGEDENT AG. Zürich. Switzerland), chlorhexidine digluconate gel 0.2% (Perio. Kin®, Laboratories KIN, Spain) and 17% EDTA solution (MD-cleanser, Meta Biomed Co. Ltd., Korea). These agents were applied to the root surface by rubbing with a micro brush, for one minute for HA and EDTA, and two minutes

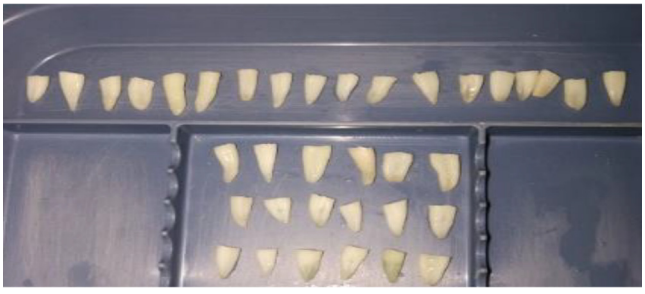

Fig. 1 Teeth preparation for the attachment of gingival fibroblasts.

for CHX. The roots were then washed once with 5 ml saline and left to air-dry.

2.2. Cell culture

Human gingival tissues were collected at the Dental Teaching Hospital, Umm Al-Qura University, from healthy adult dental patients needing crown lengthening surgery after obtaining informed consent. The gingival tissue was cut into small pieces and incubated with 3 mg/ml collagenase type I (Sigma, USA) for one hour at 37 °C. Single-cell suspension was obtained by passing cells through a 70 μM cell strainer and cultured in Dulbecco Modified Eagle Medium (DMEM, UFC Biotech, KSA) containing 10% fetal bovine serum (FBS; HyClone Thermo Scientific, USA), 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma, USA) and incubated in a humidified incubator with 5% CO_2 at 37 °C. The gingival fibroblasts at passage three (Fig. 2) were frozen until use. The roots were placed in a 24-well culture plate, one root per well, and GF were seeded on each root at a density of 2×10^5 cells/well and incubated for 48 h to allow the cells to attach to the roots. After the incubation time, each root with attached fibroblasts was transferred to a new well of 24-well plate and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay was performed to assess the viability of fibroblasts attached to the root surface (Fig. 3A) as shown previously (Talebi-Ardakani et al., 2017).

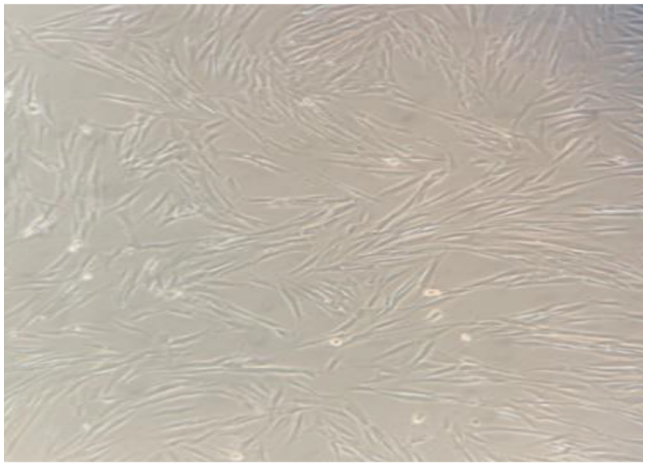

Fig. 2 A microscopic image of cultured gingival fibroblasts at 100× magnification.

2.3. Cell viability MTT assay

The MTT assay is a non-radioactive colorimetric assay for measuring cell proliferation and cytotoxicity (Carmichael et al., 1987; Mosmann, 1983). MTT enters viable eukaryotic cells and is reduced to formazan granules, which correlate directly to the number of metabolically active cells. In brief, the fibroblasts attached to the roots were transferred to a new 24-well plate and the medium was changed to a fresh medium containing 0.5 mg/ml MTT (ThermoFisher Scientific, USA) and incubated for three hours at 37 °C. The medium was removed at the end of the incubation period, and DMSO: isopropanol (1:1) solvent solution was added to dissolve formazan crystals for 30 min. The solution was transferred to a 96-well plate at 100 μl/well (Fig. 3B) and the optical density (OD) of each well was measured using a spectrophotometric microplate reader (SpectroStar Nano, BMG Lab) at a 570 nm wavelength.

2.4. Statistical analysis

GraphPad Prism7 (GraphPad Software, Inc., San Diego, CA) was used for statistical analysis. The cell viability experiments were done in triplicate and the results were expressed as mean ± standard error of mean (SEM) and analyzed by *t*-test and one-way analysis of variance (ANOVA). $P \leq 0.05$ indicates significant difference.

3. Results

In this study, the scaled roots were treated with HA or 17% EDTA or 0.2% CHX gel or left untreated. Unscaled and untreated roots were used as control. Gingival fibroblasts were seeded on the top of each root and incubated for 48 h and the viability of fibroblasts attached to the root surface was assessed using MTT assay.

The mean and standard error of mean (SEM) of cell viability of gingival fibroblasts attached to the root surfaces are shown in Table 1. The statistical significance for the difference between the scaled groups and non-scaled control group are

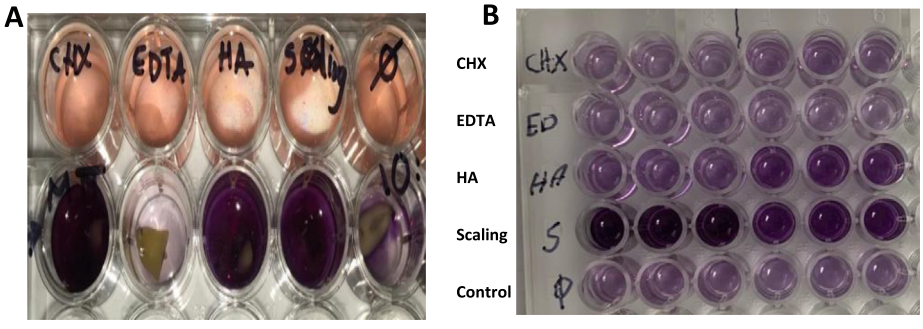

Fig. 3 Cell viability assay of the gingival fibroblasts attached to the root surface. MTT was added to fibroblasts attached to the roots in a 24-well plate (A), then the formazan crystals were dissolved. The solution was transferred to a 96-well plate (B) for reading by spectrophotometric microplate reader to assess the viability of gingival fibroblasts attached to root surface.

Table 1 Mean and standard error of mean (SEM) of cell viability of gingival fibroblasts attached to root surfaces. P-value was calculated by unpaired *t* test. $P \leq 0.05$ indicates significant difference between the scaled groups and the non-scaled control group.

Groups	Mean ± SEM	p-value	Significance vs Control
Non-scaled control	0.3107 ± 0.02187		
Scaled only	1.105 ± 0.06038	0.0002	Yes***
Scaled + HA	0.724 ± 0.02095	0.0002	Yes***
Scaled + EDTA	0.2107 ± 0.004631	0.011	Yes*
Scaled + CHX	0.334 ± 0.0205	0.4798	NS

NS = Not Significant ($P > 0.05$); asterisks denote the levels of significance (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$).

shown in Table 1, while Table 2 shows the difference between scaled groups treated with different conditioning agents and the scaled-only group.

The cell viability of gingival fibroblasts attached to the scaled-only roots was the highest, with a mean and standard error of mean of (1.105 ± 0.060) as shown in Table 1. There was a highly significant increase in fibroblast attachment to scaled-only roots compared with the cells attached to scaled roots treated with HA, EDTA, CHX or untreated control groups ($P < 0.05$).

Within the groups that received root conditioning agents (Fig. 4), HA resulted in higher cell viability compared to scaled roots treated with EDTA, CHX or non-scaled control groups ($P < 0.05$). As can be seen in Fig. 4, application of CHX on scaled roots showed higher cell viability than EDTA ($P < 0.05$) but did not lead to a significant difference in cell viability when compared with the non-scaled control group ($P > 0.05$). On the other hand, the scaled roots treated with EDTA reduced cell viability significantly compared with the non-scaled control group ($P > 0.05$).

4. Discussion

This study was carried out to evaluate the indirect effect of different conditioning materials on the viability of human gingival fibroblasts and the direct effect on their attachment to the root surface.

Table 2 Mean and standard error of mean (SEM) of cell viability of gingival fibroblasts attached to root surfaces. P-value was calculated by unpaired *t* test. $P \leq 0.05$ indicates significant difference between the scaled groups treated with different conditioning agents and the scaled only group.

Groups	Mean ± SEM	p-value	Significance vs scaled only
Scaled only	1.105 ± 0.06038		
Scaled + HA	0.724 ± 0.02095	0.004	Yes**
Scaled + EDTA	0.2107 ± 0.004631	0.0001	Yes***
Scaled + CHX	0.334 ± 0.0205	0.0003	Yes***

NS = Not Significant ($P > 0.05$); asterisks denote the levels of significance (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$).

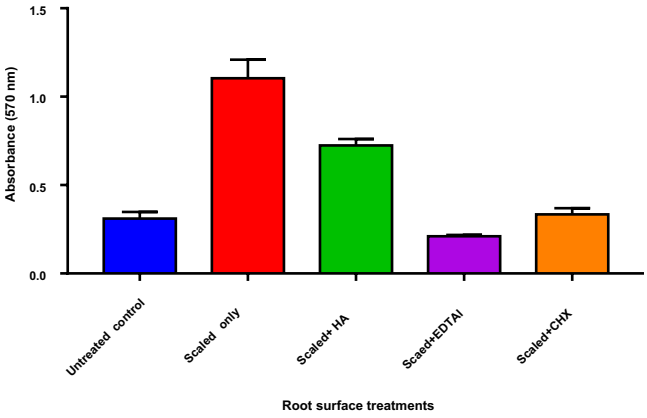

Fig. 4 Cell viability of the gingival fibroblasts attached to the root surface by MTT cell viability assay. Results are representative of two experiments. The roots were left untreated (control) or scaled. The scaled groups were treated with hyaluronic acid (HA), EDTA or chlorhexidine (CHX).

The results of the current study showed that the cell viability of the fibroblasts attached to roots treated with scaling only was the highest compared with all other groups, and that scaled teeth treated with EDTA or CHX did not enhance cell attachment. This is in agreement with other studies which demonstrated that SRP is the standard requirement for attachment of fibroblasts and that root treatment with EDTA did not enhance cell viability or attachment over SRP alone when measured using either stereomicroscope or scanning electron microscope (Girotra and Vandana, 2014; Lima et al., 2016). Additionally, other researchers stated that the exposure of fibroblasts to ≥0.02% CHX reduced the survival rate of the cells in vitro (Liu et al., 2018). Furthermore, the application of 0.5% CHX as a root conditioning agent showed less removal of the smear layer than 24% EDTA and subsequently less cell attachment (Lee et al., 2010). Other studies have reported that there was more fibroblast attachment on the root surface when 24% EDTA was used compared with 5% EDTA, and no fibroblast attachment on the root surface when saline alone was used (Babay, 2001; Belal et al., 2012). EDTA can selectively eliminate minerals from the dentin surface and expose more collagenous structures which create a favorable root surface for cell reattachment when compared with other agents or SRP alone (Blomlöf, 1996; Silva et al., 2016). Enhancing cell attachment is dependent on the ability of EDTA to completely remove the smear layers and expose the collagen matrix. Different concentrations used and variation in application time could explain the difference in the results obtained (Babay, 2001; Gamal and Mailhot, 2003; Silva et al., 2016). In this study, HA increased fibroblast attachment to the root surface significantly compared with the other conditioning agents. The application of HA modifies the surface texture of dentin via increasing surface roughness, which subsequently enhances cell attachment and spreading onto the dentin surface (Mueller et al., 2017). Although surface roughness measurement was not evaluated in this study, optimum surface roughness is needed for the attachment and proliferation of regenerative cells. The biocompatibility of HA and its ability to maintain viability, increase proliferation and migration of primary oral fibroblasts was confirmed in previous studies (Asparuhova et al., 2019). Surface irregulari-

ties, on the other hand, may provide an ideal environment for bacterial adhesion and biofilm accumulation (Ota-Tsuzuki et al., 2009). However, HA showed a bacteriostatic effect on several periodontal pathogens including *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella oris* (Pirnazar et al., 1999; Rodrigues et al., 2010). In addition, HA mouthwash was as effective as chlorohexidine in reducing plaque accumulation in periodontally healthy subjects (Rodrigues et al., 2010) and proved to be an effective plaque control agent.

The direct and/or indirect effect of HA on wound healing, inflammatory response, and periodontal pathogens seems to be supported by clinical findings where local delivery of 0.8% HA gel as an adjunct to for patients with chronic periodontitis significantly enhanced the periodontal criteria including plaque and gingival indices, pocket depth and attachment level compared with SRP alone (Shah et al., 2016). However, further long-term studies with controlled standards such as HA concentration, application time and methods are needed for better judgment of the clinical effects of HA on periodontal therapy.

This study has some limitations, and one of them is that MTT assay was used to examine the proliferation of attached cells and this was not supported by scanning electron microscope. Also, this is an in vitro study in which the effect of several confounding factors was not evaluated.

5. Conclusion

This study concluded that root scaling alone resulted in higher cell attachment and the use of root conditioning agents did not show any advantages over scaling alone. Among the conditioning agents, HA showed superior results. Further studies are needed to investigate the effect of root conditioning agents on periodontally diseased teeth in vitro and compare these agents in the clinical setting.

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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