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Early root surface colonization by human periodontal ligament fibroblasts following treatment with different biomaterials

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Abstract

Objectives. The present *in-vitro* study examined the effects of different biomaterials on early root surface colonization by human periodontal ligament (PDL) fibroblasts using confocal-laser-scanning-microscopy (CLSM). ***Materials and methods.*** Fifteen periodontally-diseased teeth were extracted, treated with scaling/root planing and longitudinally cut to obtain 30 root fragments. Fragments were treated either with 24% EDTA following application of enamel matrix derivative (EMD), 24% EDTA or EMD only, nanocrystalline hydroxyapatite (NHA) paste or oily calcium hydroxide suspension (OCHS) for 1 h each. The analogue untreated root specimens served as controls. Root fragments were incubated with human PDL fibroblasts and cellular proliferation and morphology were evaluated after 1, 3, 5 and 8 days using CLSM-visualization and image recognition software. ***Results.*** The rate of cellular proliferation was different among treatment modalities examined ($p = 0.019$). Except treatment with NHA paste all treatment modalities improved cellular proliferation on root surfaces at all different points of time compared with the control specimens. A significant difference between treatment modalities was observed between EMD and NHA paste ($p = 0.008$). No synergistic effect could be demonstrated comparing root surface conditioning with 24% EDTA and EMD application compared to 24% EDTA or EMD application only. ***Conclusion.*** The present results suggest that initial root surface colonization by PDL fibroblasts may be enhanced by root surface conditioning with 24% EDTA and application of EMD, application of 24% EDTA or EMD alone and OCHS. The addition of 24% EDTA for root surface conditioning prior to EMD application provided no synergistic effects in terms of early root surface colonization by PDL fibroblasts.

Key Words: *biomaterials, periodontal ligament fibroblasts, periodontal regeneration, proliferation, root surfaces*

Introduction

The ultimate goal of periodontal therapy is to control periodontal infection and to regenerate lost periodontal supporting tissues to their original form and function. This process requires the formation of new periodontal ligament, new cementum with inserting periodontal ligament fibers and new alveolar bone. Numerous therapeutic approaches for periodontal regeneration have been advocated in the literature, including the use of bone grafts or bone substitutes, root surface conditioning, guided tissue regeneration (GTR), growth factors or combined approaches [1,2]. The clinical outcomes following utilization of specific biomaterials to treat periodontal defects demonstrated increased attachment levels and radiographic bone fill when compared to open flap debridement alone [3]. However, different rates of success were observed throughout studies demonstrating that a complete and predictable regeneration of periodontal tissues is still difficult to obtain [4].

At the cellular level, the process of periodontal tissue regeneration requires selective migration (chemotaxis), proliferation and differentiation of cells

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from periodontal ligament (PDL) tissue. For these purposes a biocompatible root surface suitable to cell repopulation is of particular importance. The periodontally-involved root surface contaminated with periodontal microbiota and/or their endotoxins appears to be unfavorable for recolonization with cells responsible for periodontal regeneration [5]. Thus, it has been demonstrated that diseased cementum inhibits connective tissue cell attachment and proliferation [6,7]. Therefore, disinfection and modification of the exposed root surface are necessary to restore its biocompatibility and to improve cell attachment and formation of new fibrous adhesions [8]. However, mechanical instrumentation alone is not effective in removing the etiologic contaminants from the root surface, leaving a smear layer, which may inhibit re-attachment of cells to the root surface [9]. Therefore, root surface demineralization with different etching solutions such as citric acid and tetracycline hydrochloride or coating with biological mediators have been introduced [10]. The biological concept behind the demineralization procedure was to expose collagen fibers and to improve connective tissue attachment [11]. However, this procedure did not yield predictable regeneration and often resulted in root resorption and ankylosis [12]. More recently, 24% EDTA solution at neutral pH was introduced as an etching solution [13]. The treatment of root surfaces with 24% EDTA solution improved the root surface for early periodontal cell migration, attachment and orientation [14]. Although root surface demineralization may have a positive effect on wound healing it cannot be classified *per se* as a regenerative procedure. Thus, it has been demonstrated that the use of demineralizing agents to modify the root surface provides no benefit of clinical significance in regenerative treatment of patients with chronic periodontitis [15]. Currently, demineralizing agents are used as a component of regenerative procedures, e.g., prior to application of bone grafts or enamel matrix proteins.

One approach to periodontal regeneration utilizes grafting materials in osseous periodontal defects. More recently, the use of biological mediators has become the focus for regenerative techniques [12,16]. In spite of several studies evaluating the role of biomaterials in periodontal regeneration [2,3,17], fragmentary knowledge exists about their influence on the interaction between human PDL cells and the periodontally diseased root surface.

Therefore, the aim of this study was to evaluate if periodontally involved root surfaces treated with different biomaterials affect the growth of human PDL cells. In addition, we examined the influence of root surface conditioning with EDTA prior to application of enamel matrix proteins to diseased root surfaces.

Materials and methods

Biomaterials examined

Three commercially available biomaterials which are used routinely to promote periodontal regeneration were used in the present study: (1) nanocrystalline hydroxyapatite paste (NHA) (Ostim®, Heraeus Kulzer; Germany), (2) oily calcium hydroxide suspension (OCHS) (Osteora®, DFS Diamon GmbH, Germany) and (3) enamel matrix derivative (EMD) (Emdogain®, Straumann, Switzerland).

Root specimen preparation and treatment

A total of 15 single-rooted human teeth extracted for periodontal reasons, without history of any previous treatment, were obtained from the dental clinic. Following extraction, residual blood, saliva and other debris were removed by rinsing with sterile saline solution and brushing. The root surfaces were then scaled and root planed using ultrasonic and hand instruments (Hu-Friedy, Chicago, IL). Instrumentation was performed by the same experienced operator until root surfaces were adequately debrided and planed. It was attempted to prepare the specimens so that the surface was roughly plane. Subsequently, in all specimens the root was separated from the crown with a horizontal cut at the level of the notch using a water-cooled high speed bur (Busch, Engelskirchen, Germany). All specimens were then sectioned longitudinally to obtain two specimens from each root. A total of 30 sections were obtained. The root pulp was separated from the outer portion of root dentin by using a bur at low speed to avoid contamination. All specimens were then embedded in a dual-cure, automix composite core build-up material (LuxaCore® Dual, DMG, Germany), exposing the root surface. The specimens were then sterilized by autoclaving and stored in sterile cell culture medium until starting the experiments. The specimens were randomly allocated to five experimental groups (three specimens each) as follows: (1) treatment with 24% EDTA solution for 2 min; (2) treatment with 24% EDTA solution for 2 min followed by EMD application for 1 h; (3) EMD application for 1 h; (4) NHA application for 1 h; or (5) OCHS application for 1 h. Immediately after treatment the root specimens were rinsed thoroughly with physiologic solution. For each specimen the analog untreated root specimens served as controls.

Cell culture

The human PDL fibroblasts were obtained from healthy human periodontal tissues isolated from clinically healthy third molars or premolar teeth extracted for orthodontic reasons. Prior to extraction, patients

were informed about the study and agreed to experimental use of the extracted teeth. Immediately after extraction, the teeth were stored in sterile Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA) and transferred to the laboratory for PDL cell culture. Under sterile conditions the middle portion of the root was scraped with a sharp blade to obtain PDL tissue fragments. The collected tissues were washed twice with phosphate-buffered saline (PBS) containing gentamycin to reduce subsequent bacterial contamination. Tissue explants were maintained in tissue culture flasks in the presence of DMEM containing 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) 1% fungizone (Sigma, St. Louis, MO) and 10% FBS (PAA, Pasching, Austria). Cultures were incubated in a humidified atmosphere of 5% CO_2 and 95% air. Within 2–3 weeks a confluent monolayer was obtained. During this period tissue culture medium was replaced every 2 days. Cell cultures were also tested regularly to be free of mycoplasma and cell growth was monitored by phase-contrast microscopy. All cells demonstrated a fibroblast-like morphology and a comparable rate of proliferation. After the confluence was reached cells were passaged following trypsinization with 0.05% trypsin (Invitrogen, Carlsbad, CA). Cells at passage 4–6 were used for the experiments.

CLSM specimen preparation and examination

Two root sections (treated and control specimens from the same root) were placed in each of the 35-mm culture dishes. Five milliliters of cell suspension was then added to the root samples and incubated for 1, 3, 5 and 8 days at 37°C in an atmosphere of 95% humidified air and 5% CO_2. Following incubation for the selected time periods, the living PDL fibroblasts on the root surfaces (three slices from each experimental group) were visualized by confocal laser scanning microscopy (CLSM). For these purposes the PDL cells were labeled with a fluorescent vital stain (CellTracker^{TM} Green, Invitrogen, Germany) according to the manufacturer's guidelines. After incubation for 40 min at 37°C, the samples were washed with physiological saline. The PDL fibroblasts were visualized with a confocal laser scanning microscope (Leica TCS SP2, Wetzlar, Germany) equipped with argon and HeNe lasers. A water dipping objective with magnification 10× (HCX APO L 10×/0.3 W) and 40× (HCX APO L 40×/0.8 W) was used for image acquisition. The green fluorescence dye was excited at 488 nm (argon laser) and emission was collected at 500–550 nm. At 10× magnification five CLSM images (1500 µm × 1500 µm) were taken of each root specimen to evaluate cell growth, whereas 10 images (375 µm × 375 µm) were obtained at 40× magnification for morphometric analysis. The randomly chosen locations taken of each root specimen were considered to be representative of the total surface area. An accousto-optical beam splitter was employed to optimize fluorescence detection. Synchronized CSLM images in the reflection mode with the 633 nm excitation wavelength of a HeNe laser allowed the simultaneous visualization of the root surfaces and, by superimposing the fluorescence images, proper selection of cells against residual traces.

Cell imaging software

The cell counting procedure and morphological analysis were measured by using a custom-made image recognition software (www.cellimaging.medizin.uni-mainz.de; Figure 1). This software allowed detection and counting of cells within a representative area (% surface coverage) and to calculate cell size (µm) and cell surface area (µm^2). Analysis of a series of CLSM-images was accomplished with a five-step wizard. The software allows one to upload the images and to adjust the default global threshold-value calculated by the software's wizard using Otsu's-Algorithm [18]. Small particles are discarded in a labeling process to differentiate artifacts from cells. If cells cannot be separated, the software calculates the total covered surface in square millimeters using Otsu's-Algorithm.

Statistical analysis

To incorporate the correlations of the repeated measurements into the analysis, the influence of time and the different biomaterials on the cell proliferation were analyzed by fitting a multiple linear mixed model

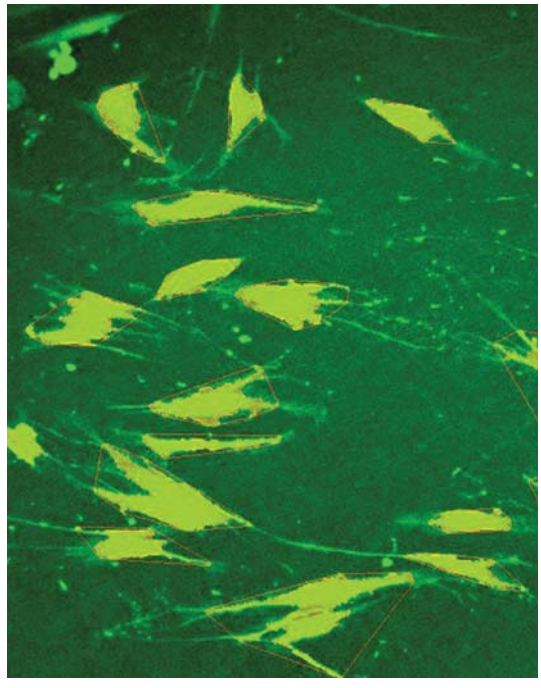

Figure 1. Detection and counting of PDL cells on the root surface with the cell observation tool. The outline of the cells is mapped by the software to calculate cell size and cell surface area.

with random intercept. The difference between the treated and untreated root specimens was calculated from the logarithmic proliferation rates and considered as a dependent variable. The logarithmic values were used due to heteroscedasticity. The fixed effects treatment and time as well as their interaction were tested as classification variables by Type Three *F*-tests. The least squares means of the outcome and their 95% confidence intervals, averaged over the effect treatment and time, respectively, were used to analyze the ratios between treated and untreated root specimens.

The two outcome variables, cell size and surface area of the morphometric analysis, were also analyzed by fitting multiple linear mixed models analogous to the cell proliferation. However, in this situation no logarithmic values were considered. The least squares means of the difference between treated and untreated root specimens, their 95% confidence intervals and related *p*-values of the two outcomes are shown among the treatments averaged over time.

Regarding the effect on cell proliferation, each pair of the five different treatments were compared by an approximate *t*-test to investigate the null hypothesis that the difference between the associated least squares means equals zero. The calculated *p*-values for all pairwise differences are adjusted for the 10 comparisons by the Tukey method.

All other *p*-values and confidence intervals in this paper are presented for descriptive reasons only and are not adjusted for multiple testing. All *p*-values are two-sided. The analysis was performed using the statistics software SAS version 9.2 (copyright 2000 SAS Institute Inc., Cary, NC) and the figures were drawn up using R version 2.12.1.

Results

The rate of cell proliferation was different among the examined treatment modalities of the root surfaces ($p = 0.019$). A distinct pattern of cell growth which would indicate a cytotoxic effect of root surface areas to PDL cells was not evident for any of the examined treatment modalities. The time as well as the interaction of time and treatment demonstrated an effect on cell proliferation ($p = 0.041$, $p = 0.047$). Thus, a trend was noted over time that root surface treatment enhanced cell proliferation as compared to the untreated control (Figure 2). All treatment modalities, except NHA paste application, improved cellular proliferation on root surfaces when compared with the control (Figure 3A). A significant difference between the treatment modalities could be observed only between EMD and NHA paste application ($p = 0.008$; Figure 3A). The highest overall growth rate of PDL cells was observed for root specimens treated with EMD; however, there was no significant difference when compared to root surface treatment with 24% EDTA and EMD application or 24% EDTA only. Thus, no synergistic effect could be demonstrated comparing root surface conditioning with 24% EDTA and EMD application compared to 24% EDTA or EMD application only.

When the morphology of the cells was visualized by CLSM with respect to surface area and cell size, differences were observed for all treatment modalities with the exception of NHA application when compared with the untreated control specimens ($p < 0.001$; Figures 3B and C). The untreated root surfaces of the control were only partly covered, with little variation in size and surface topography of the cells. The qualitative characteristics of cells appeared

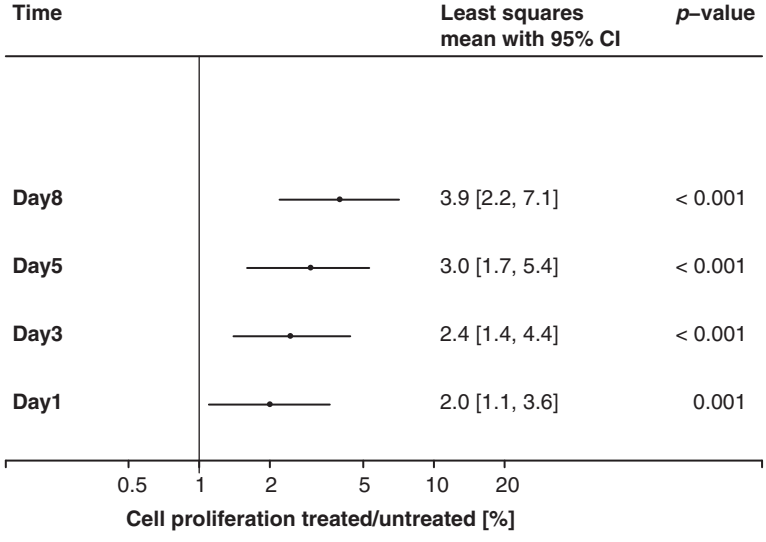

Figure 2. Influence of root surface treatment on cell proliferation after 1, 3, 5 and 8 days of incubation averaged over all treatments (*F*-test for the comparison between the four time points with $p = 0.041$).

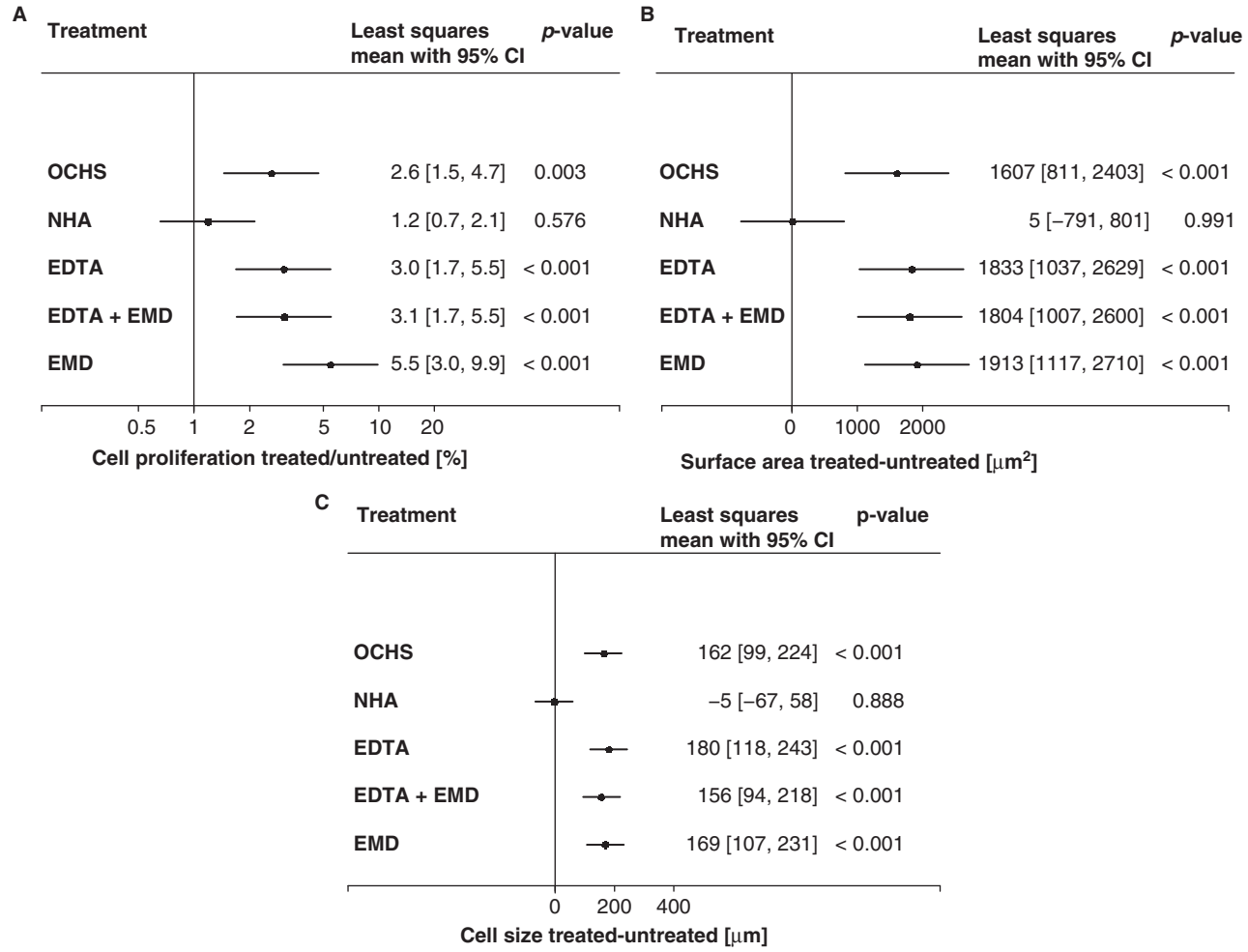

Figure 3. (A) Effect of root surface treatment with OCHS, NHA, EDTA, EMD and EDTA + EMD on cell growth (*F*-test for the comparison between the five treatments with $p = 0.019$). (B) Effect of root surface treatment with OCHS, NHA, EDTA, EMD and EDTA + EMD on the cell surface area of PDL fibroblasts (*F*-test for the comparison between the five treatments with $p = 0.011$). (C) Effect of root surface treatment with OCHS, NHA, EDTA, EMD and EDTA + EMD on the cell size of PDL fibroblasts (*F*-test for the comparison between the five treatments with $p = 0.001$).

to be enhanced following root surface treatment with EMD (Figures 4 and 5). At day 1 the root specimens were characterized by the presence of separately dispersed flat PDL fibroblasts with cytoplasmatic extensions directed to the root surfaces. After the 3 days incubation period specimens were covered with overlapped flat PDL cells in multilayers. The cells exhibited long extensions of cytoplasmatic processes and formed anastomosing networks. At day 8 a multilayer of healthy cells, well and tightly attached to the root surface, was still present (Figures 4A and 5A). Overall cells demonstrated a marked increase in size and surface area when compared to the untreated control specimens ($p < 0.001$; Figures 3B and C). Similarly, the morphology was markedly altered when cells were grown on root surfaces treated with 24% EDTA or 24% EDTA followed by EMD application (Figures 4 and 5). Thus, both groups exhibited an increase in cell proliferation over time, forming dense zones covering most of the root surface (Figures 4B and C). In both groups cells were characterized by long extensions of cytoplasmatic processes with numerous filopodia and numerous intercellular contacts (Figures 5B and C). A marked increase in cell size and surface area was observed in both groups compared to the untreated control specimens ($p < 0.001$; Figures 3B and C). In contrast, root specimens treated by NHA were characterized by the presence of a few isolated flat cells with no increase in fibroblast proliferation over time (Figure 4D). The formation of cytoplasmatic processes was observed only sporadically after 5 and 8 days incubation periods (Figure 5D). No difference to the untreated control specimens could be observed for cell size and surface area ($p = 0.888$, $p = 0.991$).

When specimens were pre-treated with OCHS cells were markedly altered in morphology (Figures 4E and 5E). Thus, already at an early stage cells exhibited thin and elongated cytoplasmatic processes and numerous intercellular contacts. The cellular body was characterized by a flattened surface which was closely adherent to the root surface. Furthermore, cells demonstrated an increase in cell size and surface

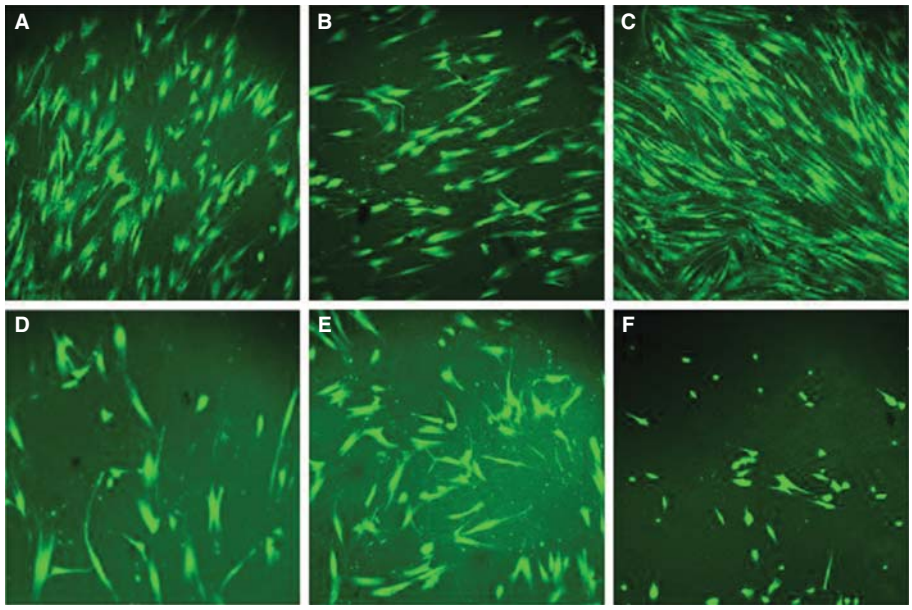

Figure 4. Fibroblast colonization on the root surface at day 8 (magnification ×10). (A) EMD-treated root surface; (B) EDTA/EMD-treated root surface; (C) EDTA-treated root surface; (D) NHA-treated root surface; (E) OCHS-treated root surface; (F) Untreated root surface; edge length 1500 μm.

area when compared to the control specimens ($p < 0.001$; Figures 3B and C).

Discussion

The formation and maintenance of a stable bond between the fibrin of the blood clot and the root surface is crucial for optimal periodontal healing [19]. However, it has been demonstrated that following mechanical debridement residual contaminating agents may impair the periodontal tissue from healing [6]. Thus, several treatment modalities including root conditioning, laser irradiation and growth factor application have been anticipated to provide a more biocompatible root surface for early cell migration, attachment and fiber development [20–22]. In this context, chemical root conditioning with demineralizing agents, such as citric acid, tetracycline and EDTA has been widely used as a therapeutic modality to modify the diseased root surface. Nevertheless,

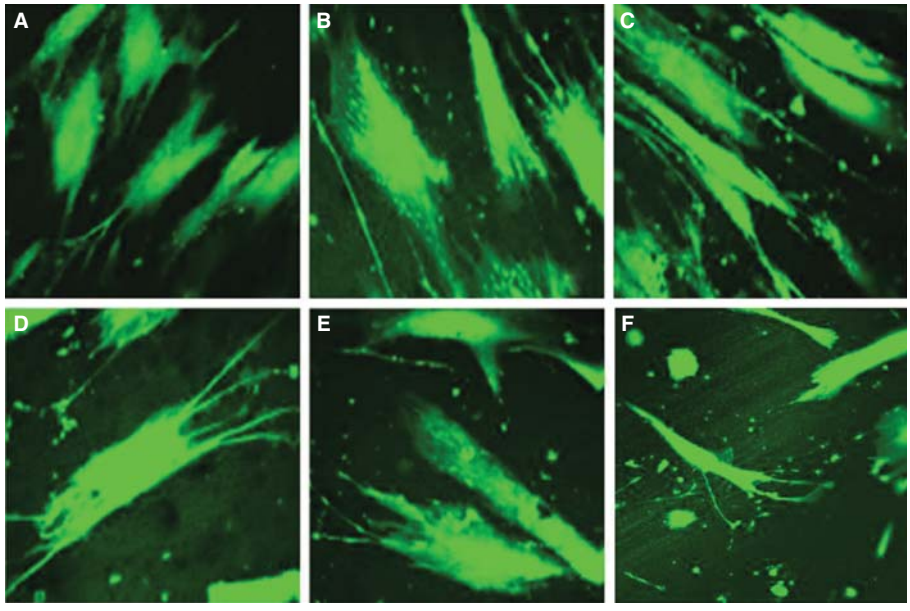

Figure 5. Fibroblast colonization on the root surface at day 8 (magnification ×40). (A) EMD-treated root surface; (B) EDTA/EMD-treated root surface; (C) EDTA-treated root surface; (D) NHA-treated root surface; (E) OCHS-treated root surface; (F) Untreated root surface; edge length 375 μm.

despite several *in vitro* studies suggesting a benefit from root surface conditioning [23,24], it has been demonstrated that root surface conditioning in humans provides no clinical advantage [15]. More recently, root surface conditioning is used as a pre-treatment prior to application of regenerative agents such as enamel matrix derivative [12]. In the present study we evaluated the effects of different therapeutic agents administered directly onto the periodontally involved root surface on the biocompatibility of PDL fibroblasts. EDTA was used as pre-treatment to study the effect of root surface conditioning on the effects of EMD.

The results demonstrated that root surface conditioning with 24% EDTA for 2 min promoted an increase in cellular density compared to the scaled and root planed diseased root surface alone. Our observations are in agreement with previous studies [14,25,26], which have demonstrated the capacity of 24% EDTA to improve the migration, attachment and proliferation of fibroblasts on diseased root surfaces. From a morphological point of view, cells were closely adherent to the root surface and characterized by long extensions of cytoplasmatic processes, supporting the report that EDTA demineralization appeared to promote early cellular colonization and subsequent tissue formation by providing a more biocompatible root surface for cell attachment [13]. In contrast, the scaled and root planed diseased root surfaces were populated by only a few cells with limited cytoplasmatic processes. The concentration and application period of EDTA was determined according to several reported observations. Thus, Babay [25] demonstrated that the re-attachment of human gingival fibroblasts to root surfaces was significantly more enhanced following conditioning with 24% EDTA than with 5% EDTA. Furthermore, results from *in vitro* studies and *in vivo* animal studies reported a more biocompatible surface for periodontal healing following treatment with 24% EDTA when compared to a mineralized root surface covered by a smear layer [13,27]. However, root surface conditinioning with 24% EDTA failed to provide any adjunctive effects clinically [28].

The root surface treatment with 24% EDTA followed by EMD application or EMD only improved cellular proliferation when compared with the scaled and root planed diseased root surface alone. The beneficial effect of EMD on human PDL fibroblast proliferation is in agreement with previous findings [29]. Our morphological observations confirmed the improved biocompatibility following root surface treatment with EMD. Thus, the attached PDL fibroblasts appeared with extensive spreading and increased size and surface area. Similar responses of PDL fibroblasts on EMD-treated root surfaces were reported by Cattaneo et al. [30]. However, application of EMD onto root surfaces previously treated with EDTA did not increase cellular density compared to EMD or EDTA application only. Thus, no synergistic effect was observed between EDTA and EMD within the 8-day period. Similarly, from a morphological point of view no differences could be observed between combined EDTA and EMD application as compared to EDTA or EMD alone. These results are supported by previous findings showing that pre-conditioning with EDTA did not alter the clinical outcomes of EMD therapy upon intrabony defects [31]. In an *in vitro* study, Silverio et al. [26] reported that pre-conditioning of the root surface with EDTA failed to improve the effect of basic fibroblast growth factor (b-FGF) on proliferation of fibroblasts, suggesting chemical incompatibility between the substances. Moreover, the authors suggested the possibility that EDTA may interfere with the cellular receptors specific to the growth factor on fibroblasts. In contrast, Belal et al. [32] reported that the application of rhPDGF-BB to root surface specimens previously treated with EDTA gel significantly enhanced PDL proliferation and/or viability compared to EDTA gel application only. However, this effect was not enhanced in comparison to the use of rhPDGF-BB alone. Although the present study failed to demonstrate a synergistic effect of EDTA and EMD, exposure of collagen fibers following root conditioning with EDTA may enhance the retention and contact of EMD over a longer period of time. Indeed, results from immunohistological studies demonstrated the presence of EMD on conditioned root surfaces for up to 4 weeks [33]. Gestrelius et al. [34] evaluated root surface colonization by periodontal ligament cells following etching with 37% phosphoric acid and EMD application. After 14 days, ~ 3/4 of the originally denuded area was covered by PDL cells, as compared to 1/8 in controls. Although the improved biocompatibility following root surface treatment with EMD was not altered by the omission of EDTA in the present study, further investigations are needed to evaluate the retention and stability of EMD binding to the root surface without previous root conditioning. Moreover, it has to be emphasized that, in a clinical setting, other possible beneficial effects of root surface conditioning with EDTA prior to EMD application have to be considered, e.g. removal of the smear layer after mechanical debridement, exposure of collagen fibers, establishment of an early fibrin linkage, antimicrobial properties, long-term stability of EMD binding and improved histological regenerative results.

The treatment of the root specimens by NHA failed to improve cell density when compared to the scaled and root planed diseased root surfaces alone. Furthermore, topical exposure of root surfaces to NHA did not affect cell morphology. These results are in contrast to previous observations, in which we reported that NHA in a soluble form promoted

PDL cell proliferation *in vitro* [35]. On the other hand, Alliot-Licht et al. [36] reported increased protein synthesis, decreased proliferation and alkaline phosphatase activity on PDL cells in the presence of HA with a particle size < 20 μm. The contrary results may be attributed to differences in preparation and concentration of NHA, but also due to different coating procedures. The adhesion and subsequent response of PDL fibroblasts may be affected by the contact domain size between the cell and the substrate. Thus, small domains of the NHA on the root surface could limit focal adhesion of the cells. Indeed, it has been demonstrated that contact domains larger than 100 nm in width are required for cell survival [37], whereas the NHA employed in the present study exhibits a mean size of ~ < 100 nm.

The present study further revealed that root surfaces treated with OCHS are more biocompatible to PDL fibroblasts for early cell proliferation when compared to periodontally diseased treated control specimens. In relation to cellular morphology, treatment of root specimens with OCHS appeared to produce flat cells firmly attached to the surface. Moreover, an increase in cell size and surface area in comparison to the control specimens was observed. Our observations of increased proliferation rates were corroborated by previous findings, where the PDL cell proliferation rate was significantly increased when OCHS was present in culture [38]. OCHS has been shown to create a stable, long-lasting pH gradient of 7–11 within the tissue without causing irritation [39]. Thus, the oily suspension produces a long-term, mild alkaline environment, because only the calcium hydroxide at the interface between the liquid/oily phase is released. Stratul et al. [40] reported in a clinical study that the treatment of intra-bony defects with OCHS significantly enhanced clinical results for probing pocket depth reduction and probing attachment gain when compared to open flap debridement alone.

It has to be pointed out that the results obtained by our experimental *in vitro* model cannot recreate the events involved in periodontal regeneration and the complex interaction of cells *in vivo*.

In sum, our findings present evidence that the treatment with 24% EDTA, EMD or OCHS improved the root surface for early periodontal cell colonization when compared to scaled and root planed diseased root surfaces alone. Furthermore, this study failed to demonstrate *in vitro* a synergistic effect between EDTA and EMD. The overall contribution of root surface conditioning with EDTA in the process of periodontal regeneration with EMD therapy needs to be determined with further studies.

Declaration of interest: No benefit of any kind will be received either directly or indirectly by the authors.

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