Stimulation of human gingival fibroblasts viability and growth by roots treated with high intensity lasers, photodynamic therapy and citric acid

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A R T I C L E  I N F O

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A B S T R A C T

Objective: The aim of this study was to compare the effect of root biomodification by lasers, citric acid and antimicrobial photodynamic therapy (aPDT) on viability and proliferation of human gingival fibroblasts (FGH).
Design: Groups were divided in control (CC – only cells), and root fragments treated by: scaling and root planing (positice control – SC), Er:YAG (ER–0.5W,15 Hz,10s,1640 nm), Nd:YAG (ND–0.5W,15 Hz,10s,1640 nm), antimicrobial photodynamic therapy (PDT–InGaAIP,30 mW,45J/cm²,30s,660 nm,toluidine blue O), citric acid plus tetracycline (CA). Fibroblasts (6th passage, 2 × 10⁶) were cultivated in a 24-h conditioned medium by the treated root fragments. Cell viability was measured by MTT test at 24, 48, 72 and 96 h. In a second experiment, FGH cells (10⁴) were cultivated on root fragments which received the same treatments. After 24, 48, 72 h the number of cells was counted in SEM pictures. In addition, chemical elements were analyzed by energy dispersive spectroscopy (EDS). Data was analyzed by two-way ANOVA (first experiment), repeated measures ANOVA (second experiment) and ANOVA (EDS experiment) tests complemented by Tukey’s test (p < 0.05).
Results: ND, PDT and CA promoted higher cell viability (p < 0.05). ND and ER groups presented higher number of cells on root surfaces (p < 0.05). ER group presented higher calcium and CA group a higher carbon percentages (p < 0.05).
Conclusions: All treatments but scaling and root planing stimulated fibroblast viability while Er:YAG and Nd:YAG treated root surfaces presented higher number of cells.

1. Introduction

One of the main challenges in periodontal treatment is how to eliminate the subgingival bacteria and to convert the root surface in a biocompatible environment. Bacterial invasion in radicular cementum and dentinal tubules reach 300 μm and elimination by mechanical treatment is challenging. Therefore adjuvant methods for root biomodification are proposed (Adriaens, Edwards, DeBoever, & Loesche, 1988). Among the options are chemical treatment (Register & Burdick, 1975; Sant’Ana, Marques, Barroso, Passanezi, & de Rezende, 2007), high-energy lasers (Feist et al., 2003; Gaspirc & Skaleric, 2007; Hamaoka, Moura-Netto, Marques, & Moura, 2009; Qadri, Javed, Poddani, Tunér, & Gustafsson, 2011) or recently, antimicrobial photodynamic therapy (Andrade et al., 2013; Kê et al., 2013; Qin, Luan, Bi, Sheng, Zhou, & Zhang, 2008; Salmeron, et al., 2013; Sgolastra et al., 2013). All of these treatments are adjunctive to scaling and root planing and show distinct advantages. These therapies also promote root surface modification and demonstrated antimicrobial effects (Andrade et al., 2013).

Hard surface demineralization has been used in periodontology since 1973, when Register showed an accelerated reattachment of fibers and cementogenesis in surgically exposed and demineralized roots in situ (Register, 1973). Various acid products were tested aiming hard tissue demineralization without side effects. The best results were obtained with citric acid, at pH 1, for 3 min (Register & Burdik, 1975). Citric acid demineralization removes smear layer, lipopolysaccharides and exposes collagen fibrils, which improves blood clot formation with higher retention of fibrin (Leite et al., 2010). The
association of tetracycline with citric acid optimizes these effects (Sant’Ana et al., 2007).

High intensity lasers (Er:YAG) promote root biomodification with efficient calculus removal, resulting in a rough surface (Feist et al., 2003; Passanezi, Damante, de Rezende, & Greghi, 2015; Aoki et al., 2000). Root surfaces treated by Er:YAG are superior for fibrin clot formation and blood cell attachment (Cekici, Maden, Yildiz, San, & Isik, 2013) and are attractive to gingival fibroblasts (Feist et al., 2003). Clinically, Er:YAG as an adjunctive treatment for periodontal disease results in reduced probing depths and gingival index (Gaspirc & Skaleric, 2007). Nd:YAG lasers promote significant black pigmented bacteria reduction in class two furcation lesions (Andrade et al., 2008). The improved root biocompatibility is due to surface modification and bactericidal effect (Hamaoka et al., 2009). Also, additional benefits were demonstrated in smokers in relation to conventional therapy for treatment of periodontal disease (Elatas & Orbak, 2012). A recent meta-analysis suggested that the adjunctive treatment with Nd:YAG to scaling and root planing is responsible for probing depth and gingival crevicular fluid reduction (Sgolastra et al., 2013).

Antimicrobial photodynamic therapy (aPDT) is indicated to lethal photosensitization of bacteria. The basis of aPDT is the activation of a photosensitizing drug as toluidine blue O (TBO) by a red laser. The chemical reaction releases oxygen reactive species capable of destroying target microorganisms. aPDT exerts an additional effect on periodontopathogenic bacteria and reduces virulence factors. This therapy is successfully associated to nonsurgical and surgical periodontal therapy modulating the extracellular matrix and bone remodeling (Andrade, Garlet, Silva, Fernandes, & Milanezi, 2013). A meta-analysis demonstrated that aPDT exerts additional benefits on periodontal treatment outcomes as probing depth reduction and clinical attachment level gain in a short-term period (Sgolastra et al., 2013). Recent researches of our group have proven that Toluidine Blue O [100 μg/ml] and pH [4.31] commonly used in aPDT causes demineralization and loss of Knoop microhardness on dentin surfaces without damage to gingival fibroblasts (Damante et al., 2016). This result could be useful for root surface decontamination and demineralization in periodontal treatments.

There is no data in literature addressing the biocompatibility of roots demineralized by aPDT with toluidine blue O. Thus, the first step is to test these effects in cell culture tests. Cell culture presents different cell type and enable the study of cell effects in cell culture tests. Cell culture presents different cell type and enable the study of cell effects.

The aim of this study was to compare the effects of root biomodification by lasers (Er:YAG, Nd:YAG) citric acid and antimicrobial photodynamic therapy on the viability and proliferation of human gingival fibroblasts.

2. Materials and methods

This research was approved by Ethical Committee on Human Research of Bauru School of Dentistry – University of São Paulo (#086/2011).

2.1. Preparation of root fragments

Sixty teeth extracted for severe periodontal disease were selected and stored in saline solution at 4 °C. Dental roots were cut with a disk at the following dimensions: 4 mm length, 2 mm width, 2 mm height. One hundred and twenty fragments were obtained and sterilized in autoclave. All fragments were scaled with Gracey curettes (Hu Friedy, Chicago, USA) (20 strokes/fragment) and washed with saline for 30 s.

2.2. Experimental groups

The experimental groups were divided as follows:

- CC – cell control (first experiment only)
- SC – scaling and root planing control
- ER – laser Er:YAG
- ND – laser Nd:YAG
- PDT – antimicrobial photodynamic therapy – Toluidine Blue O (pH 4.31) + Laser InGaAlP
- CA – citric acid + Tetracycline – pH 1

The CC group had only cells cultured in ideal conditions of growth and it was used only on the first experiment. The SC group consisted in fragments treated with 20 strokes of curette. The ER group was treated with an Er:YAG laser (Fotona, Twinlight, Slovenia) – 2940 nm, scanning mode, 60 mJ, 10pps, 10 Hz, 10s, focal distance 12 mm, diameter 0.466 mm², water spray (0.34 ml/s). The ND group was treated with a Nd:YAG laser (Fotona, Twinlight, Slovenia) – 1640 nm, contact mode, 5J, 0.5W, 15 Hz, 10s. The PDT group was treated with Toluidine Blue O in deionized water (TBO – 100 μg/ml) for 60 s and irradiated with an InGaAlP red laser (Thera Lase – D.M.C. Equipamentos Ltda, São Carlos, Brazil) – 660 nm, 30 mW, 45J/cm², 30s, sweeping mode, 0.028 cm² spot area, 360J, 1.07W/cm². The CA group was treated with a gel containing 50% citric acid plus 10% tetracycline (pH1). After all treatments, fragments were washed with saline solution for 30s.

2.3. First experiment – conditioning of the medium with treated root fragments

Cells used in this study were human gingival fibroblasts (FGH lineage). This lineage was obtained by primary culture and was stored in liquid nitrogen. Cells were cultured in a conventional Dulbecco Modified Eagle’s medium (DMEM) supplemented by 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Penicillin 10.000 UI, streptomycin 0.050 g/L, Amphotericin B) in a humidified air–5% carbon dioxide (CO₂) atmosphere. Cells from the 6th passage were used for the experiment in a concentration of 2 × 10⁴ cells/well in 96-well plates. The experimental groups were in sextuplicate.

A conditioned medium was prepared by submerging the treated root fragments (10/group) in conventional DMEM (supplemented by 10% FBS and 1% antibiotic-antimycotic solution (Penicillin 10.000 UI, streptomycin 0.050 g/L, Amphotericin B). After preparation, the conditioned medium was maintained in incubator for 24 h following the ISO standard 10993-12 (International Organization for Standardization, 1996). After 24 h, root fragments were removed and the conditioned medium was used to substitute the conventional medium in the wells with the exception of CC group.

The cell viability was measured after 24, 48, 72 and 96 h by MTT assay. The MTT assay measures cell mitochondrial activity involving the conversion of the water-soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to an insoluble formazan. The formazan is then, solubilized, and the concentration determined by optical density (OD) at λ 570 nm.

Results were statistically analyzed by a software Statistica 11.0 (www.statsoft.com/company). The Two-way ANOVA complemented by Tukey’s test was applied (p < 0.05).

2. Second experiment – SEM analysis of cell growth on treated root surfaces

Human gingival fibroblasts were cultured as described above. Cells from the 6th passage were used for the experiment in a concentration of 10⁴ cells/well in 24-well plates. The experimental groups were in triplicate. After treatment, the root fragments were sterilized and placed in 24-well plates. FGH cells were plated over the fragments and cultured in DMEM (supplemented by 10% FBS and 1% antibiotic-antimycotic solution) for 24, 48 and 72 h.

Samples were prepared for SEM analysis by fixation in Karnovsky fixative and dehydrated in ethanol staging to 100% and embedded in glycol methacrylate. Thin sections were stained with 1% uranyl acetate and lead citrate. Observations were performed in a Zeiss transmission electron microscope at 80 kV.
solution and post-fixation in 1% osmium tetroxide in the 0.05 M cacodylate buffer solution. After dehydration in serial dilutions of acetone, specimens were spatter coated with gold and analyzed in a scanning electron microscope (Personal SEM Express – Delmont Pennsylvania, USA). Cell counting was performed by a single calibrated blinded examiner (CAD) in the electronmicrographs of five defined areas in each root fragment as described in literature (Feist et al., 2003). The total number of cells for each group was obtained for each experimental period (24, 48, 72 h).

Results were statistically analyzed by a software Statistica 11.0 (www.statsoft.com/company). The repeated measures ANOVA complemented by Tukey’s test was applied (p < 0.05).

2.5. Third experiment – energy-dispersive spectroscopy (EDS) analysis

Three samples of each group (without cells) were analyzed by energy-dispersive spectroscopy (EDS). The spectra for EDS measurements were obtained for 100 s lifetime (voltage: 15 kV). Atomic percentage (A%) of the following chemical elements were analyzed: carbon (C), oxygen (O), magnesium (Mg), phosphorus (P), sulfur (S), and calcium (Ca).

Results were statistically analyzed by a software Statistica 11.0 (www.statsoft.com/company). The ANOVA complemented by Tukey’s test was applied (p < 0.05).

3. Results

3.1. First experiment – conditioning of the medium with treated root fragments

Cell viability is presented in optical density units (OD) (Table 1). Cell viability between CC group and SC group presented no statistical significant difference (p > 0.05) at all periods. At 24 h, there was no statistically significant difference among groups (p > 0.05). At 48 h cell viability was significantly stimulated by PDT (OD = 0.454) and CA (OD = 0.261) in relation to other groups (p < 0.05). At 72 h there was a significant increase in cell viability for ND group (OD = 0.809) and PDT (OD = 0.623) in relation to control (CC and SC) and ER groups (p < 0.05). At 96 h cell viability was significantly stimulated by CA group (OD = 0.689) in relation to other groups (p < 0.05). At 96 h there was a decrease in cell viability in ND and PDT groups, in relation to 72-h period (p < 0.05). It occurred by cells reaching confluence in those wells. ND and PDT groups at 72 h and CA group at 96 h presented the highest cell viability numbers (p < 0.05).

3.2. Second experiment – SEM analysis of cell growth on treated root surfaces

The results of the second experiment are presented in Table 2. At 24 h, there was a higher number of cells at Er:YAG and Nd:YAG groups (p < 0.05). At 48 h the number of cells was similar to all groups (p > 0.05). At 72 h, the Nd:YAG group exhibited the highest number of cells, but it was statistically similar to all groups (p > 0.05) but CA group. Considering all groups and all periods, ND and ER had a significant higher number of cells at 24h-period (p < 0.05). Fig. 1 illustrates one representative SEM image of each group at 24, 48 and 72 h.

3.3. Third experiment – energy-dispersive spectroscopy (EDS) analysis

The results of EDS analysis are presented in Table 2. Citric acid plus tetracycline (CA) promoted surface demineralization, with an increase in organic content (% carbon) (p < 0.05). Er:YAG laser promoted a significant increase in calcium content in relation to control group (p < 0.05). This may be explained by the ablation effect, which may promote a calcium spreading on the root surface. ND and PDT were similar to control group in all the elements analyzed (p > 0.05).

4. Discussion

Results demonstrated a positive influence of root surface biomodification with high-energy lasers, aPDT and citric acid plus tetracycline in a fibroblast lineage viability. In the first experiment, root surface treatment with Nd:YAG, aPDT and citric acid plus tetracycline enhanced cell viability in later periods of time. In the second experiment, surfaces treated with Er:YAG and Nd:YAG promoted a higher cell adhesion and growth. Root fragments treated by Er:YAG exhibited a higher calcium content on the root surface, while citric acid plus tetracycline presented a higher carbon content due to demineralization.

It is important to highlight that the aim of this study was to evaluate how the root surface biomodification by different treatments (laser and acid substances) could enhance fibroblast growth. After surgical procedures as root coverage, gingival fibroblasts are the first cells in contact to root surface, and the root biomodification may favor the new insertion of collagen fibers (Zeite et al., 2010; Register, 1973).

About four decades, root conditioning with acid substances has been used in periodontics (Register & Burdik, 1975). Root conditioning with citric acid usually show beneficial results in cell culture (San’Ana et al., 2007; Negi, Krishnamurthy, Ganji, & Pendor, 2015), animal (Aoki et al., 2015; Romanos, Everts, & Nentwig, 2000) and human studies (Giannini et al., 2006; Soukos, Wilson, Burns, & Speight, 1996). In the first experiment of the present study, citric acid plus tetracycline was capable of stimulating cell viability until 96-h period. In the second experiment, the number of cells on root fragments was similar to the

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>CC</th>
<th>SC</th>
<th>ER</th>
<th>ND</th>
<th>PDT</th>
<th>CA</th>
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<tbody>
<tr>
<td>24h</td>
<td>0.065±0.01</td>
<td>0.052±0.01</td>
<td>0.014±0.01</td>
<td>0.080±0.03</td>
<td>0.061±0.03</td>
<td>0.093±0.03</td>
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<tr>
<td>48h</td>
<td>0.129±0.10</td>
<td>0.190±0.11</td>
<td>0.138±0.10</td>
<td>0.125±0.10</td>
<td>0.454±0.13</td>
<td>0.263±0.12</td>
</tr>
<tr>
<td>72h</td>
<td>0.246±0.11</td>
<td>0.425±0.16</td>
<td>0.560±0.20</td>
<td>0.809±0.19</td>
<td>0.623±0.13</td>
<td>0.520±0.11</td>
</tr>
<tr>
<td>96h</td>
<td>0.274±0.20</td>
<td>0.320±0.13</td>
<td>0.475±0.21</td>
<td>0.501±0.17</td>
<td>0.481±0.10</td>
<td>0.689±0.14</td>
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Optical Density ± standard deviation. Different letters = p < 0.05 (comparisons of experimental groups to controls).
Fig. 1. Scanning electron microscopy images of different groups and periods. SC = scaling control (A,B,C); CA = citric acid plus tetracycline (D,E,F); ER = Er:YAG laser (G,H,I); ND = Nd:YAG laser (J,K,L); PDT = antimicrobial photodynamic therapy (M,N,O); at 24 (A,D,G,J,M), 48 (B,E,H,K,N) and 72 h (C,F,I,L,O). Arrows indicate fibroblasts. Original magnification 500×.

Fig. 2. Atomic percentage (A%) of the chemical elements: carbon (C), oxygen (O), magnesium (Mg), phosphorus (P), sulfur (S), and calcium (Ca), obtained by EDS. * = p < 0.05 in relation to other groups.
other groups showing no additional effect of this therapy. This result is not in accordance with others, where citric acid plus tetracycline enhanced cell growth on root fragments (Sant’Ana et al., 2007). Possibly, remnants of acid or tetracycline at the root surface impaired cell growing.

More recently, with the popularization of laser in dentistry, root biomodification by high-energy lasers has been extensively studied (Feist et al., 2003; Hamaoa et al., 2009). Both in vitro and in vivo irradiation with Nd:YAG and Er:YAG lasers exhibited positive results in cell proliferation and enhancement of periodontal clinical parameters (Gaspir & Skaleric, 2007; Qadi et al., 2011). In the second experiment, Er:YAG promoted a significant increase in cell numbers at 24 h and this result is in accordance to literature where the Er:YAG treatment was superior in stimulating fibroblast adhesion and proliferation. It could be suggested that the higher content of calcium on the surface, promoted by erbium ablation has a beneficial effect for cell growth. Alternatively, the rough surface promoted by laser ablation may be more attractive to fibroblast adhesion.

In this study, Nd:YAG treatment promoted higher cell viability at 72 h in the first experiment and a higher number of cells comparable to Er:YAG at 24 h (second experiment). These results are in accordance to Negi et al. (2015), that revealed a higher number of mouse fibroblasts on root fragments treated with scaling and root planing plus Nd:YAG (Negi et al., 2015). There is some controversy in literature about Nd:YAG application, because it is a deeply penetration laser which scatters deeply in the tissue. According to its settings, the laser can damage dental structure because part of the emitting light is converted into heat by refraction or diffused reflection at the tip end, creating a condition called “hot tip” (Aoki, et al., 2015). It is important to set a lower power in order to avoid damage the tissue. In the present study, we used 0.5W. There are reports about an extensive melting and damage in titanium implants with Nd:YAG irradiation with power settings at 2, 4 and 6W (Romanos, Everts & Nentwig, 2000). On the other hand, studies that used up to 1.4W reported no damage at the titanium surface (Giannini et al., 2006). This result could be extrapolated to root surfaces, where such heating may damage pulp and carbonize hard dental tissues.

The central idea of adding an antimicrobial photodynamic therapy group in the present study arose from previous results of our group (Damante et al., 2016). In an in vitro model, the toluidine blue O solution (100 μg/ml) caused a significant loss of dentin microhardness and a surface wear of 9.06 ± 7.69 μm (19). This can be an undesired effect, but in Periodontics, these effects may be as good as or similar to citric acid conditioning to stimulate cell adhesion and proliferation. Our first concern was to evaluate if the cell viability would be diminished in PDT group because some studies showed a decrease in keratinocytes and fibroblasts viability of 12% and 8% with low concentrations of toluidine blue O (TBO) (Soukos et al., 1996). The authors credited this toxicity to the uptake of TBO of 7.5% by keratinocytes and 6.9% by fibroblasts. This fast uptake of TBO and its binding to DNA and RNA may explain its cytotoxic effects (Soukos et al., 1996). In relation to this last study, we have to consider that the TBO was used in cell culture environment, which may disturb cell growth. Clinically, the tissues buffer effect and gingival crevicular fluid may rapidly neutralize or eliminate these dyes from treatment site. In the present study, aPDT increased fibroblast viability after 72 h of culture as ND group. In addition, the number of cells and chemical elements on the surface of roots treated by PDT were similar to control. These results confirm data in literature regarding the safety of the technique, which demonstrates that aPDT is toxic for bacteria but is safe for cells (Maisch, 2007). Moreover, histological studies revealed no inflammation in tissues after contact with blue dyes (Qin et al., 2008; Salmeron et al., 2013). A study by Kömerik et al. (2013) showed no adverse effects as ulcer formation on the epithelium or inflammation in the connective tissue of rats after aPDT even with the highest light doses and toluidine blue concentrations (Kömerik et al., 2013).

This is the first study in literature to use treated root fragments to condition cell culture medium. This methodology was based on ISO standard 10993-12 that preconizes to incubate the material at 37 °C for 24 h at a ratio of 60 cm² surface per 20 ml of cell culture medium (International Organization for Standardization, 1996). We may suggest that some ions and chemical elements as calcium and phosphorus released in the medium can be responsible to stimulate cell growth and that is the subject of our future researches. In order to discover which elements could be released from treated root fragments, we performed the third experiment with EDS. Indeed, in the present study, the EDS indicated a higher calcium content on the root surface treated by Er:YAG and a significant increase in carbon content in surfaces demineralized by citric acid plus tetracycline. Calcium is an important cellular signaling ion. It controls a multitude of biological processes and activate cascades of signaling pathways (Decrock et al., 2017). Many organelles store calcium ions as endoplasmic reticulum and mitochondrial (Decrock et al., 2017). Moreover calcium ions are involved in ATP release (Decrock et al., 2017). Although the calcium intake by cells depends on membrane channels, an environment with a high concentration of calcium may stimulate cell proliferation and metabolism.

Considering the limitations of an in vitro study, we could suggest an extrapolation to clinical application. Therefore, laser treatment in surgical periodontal procedures could be an excellent choice to induce fibroblast proliferation and soft tissue healing.

5. Conclusion

All treatments, with the exception of scaling and root planing stimulated the viability of human gingival fibroblasts. Although Er:YAG treatment resulted in a higher calcium content on the root surface, the cell proliferation was similar to Nd:YAG.

Competing interests

Authors declare no conflict of interest.

Ethical approval

This research was approved by Ethical Committee on Human Research of Bauru School of Dentistry – University of São Paulo (#086/2011).

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References

attachment on Er: YAG laser applied root surface using scanning electron microscopy. 

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