

Adhesion and Proliferation of Human Gingival Fibroblasts on Root Surfaces following Photodynamic Therapy: An In Vitro Study

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Abstract

Background and Aim: Previous studies have shown that some dyes used in photodynamic therapy (PDT) have a low pH and may cause root demineralization and remove the smear layer. This study aimed to assess and compare the effects of root biomodification with citric acid and PDT on the adhesion and proliferation of human gingival fibroblasts (HGFs).

Materials and Methods: In this in vitro experimental study, 75 single-rooted teeth extracted due to periodontal disease were assigned to the following treatment groups: (I) Scaling and root planing (SRP) alone; (II) SRP + citric acid (pH=1, 60 s); (III) PDT with toluidine blue (TB; 635 nm laser, 150 mW for 30 s); (IV) PDT with methylene blue (MB; 660 nm laser, 150 mW for 30 s) and (V) PDT with indocyanine green (ICG; 810 nm laser, 150 mW for 30 s). Fibroblasts were then cultured in Dulbecco's modified Eagle's medium along with the treated root pieces. Adhesion and proliferation of fibroblasts were quantified using the methyl thiazolyl tetrazolium (MTT) assay at 24, 48 and 72 hours. Data were analyzed using SPSS version 22 by repeated measures ANOVA. Pairwise comparisons were performed by the LSD test.

Results: Adhesion and proliferation of HGFs significantly increased in all groups from 24 to 72 hours ($P=0.0001$). There was no significant difference in this respect among the groups at the three time points ($P=0.143$).

Conclusion: PDT did not have any adverse effect on the adhesion and proliferation of HGFs on dentin, which confirms the safety of this treatment modality.

Key Words: Photochemotherapy, Lasers, Fibroblasts, Cell Adhesion, Cell Proliferation

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Introduction

The conventional treatment of periodontally-involved teeth is based on mechanical

debridement (1,2), which may not be able to fully eliminate the infection due to the formation of smear layer (3,4). Bacteria

penetrate deep into radicular cementum and dentinal tubules by up to 300 μm depth; thus, they cannot be eradicated by mechanical treatment alone (5). Therefore, adjunct techniques for root biomodification were proposed such as chemical treatment using citric acid (6), high-vitality lasers (7) and recently, antimicrobial photodynamic therapy (aPDT) (8). These strategies are proposed as an adjunct to scaling and root planing (SRP). They modify the root surface and exert antimicrobial effects (9).

The aPDT involves the utilization of a photosensitizer that is irradiated with proper wavelength of light to create free radicals and reactive oxygen species such as singlet oxygen with bactericidal and fungicidal activities (10, 11). Since aPDT eliminates the microorganisms lodged in hard-to-reach areas of periodontal pockets, it is used as an adjunct for treatment of gingivitis, periodontitis and peri-implantitis (12). Recently, this strategy was proposed to remove the smear layer. Damante et al. (8) utilized PDT with methylene blue (MB) for root modification in vitro, and evaluated the adhesion and proliferation of fibroblasts to dental surfaces. However, it was less effective than citric acid.

Since aPDT is known as an adjunct treatment in periodontal therapy, its effect on attachment and proliferation of gingival fibroblasts can be used to improve cell adhesion. As of today, different photosensitive materials are available for use as photosensitizer in aPDT, but adequate information is not available on their efficacy. This study aimed to assess the efficacy of MB, toluidine blue (TB) and indocyanine green (ICG) for smear layer removal and their effects on adhesion and proliferation of human gingival fibroblasts (HGFs) in vitro.

Materials and Methods

This in-vitro experimental study evaluated 75 single-rooted teeth extracted due to periodontal disease. All teeth underwent scaling with an ultrasonic scaler and root planing with Gracey curettes (Hu Friedy, Chicago, USA). Next, 5 \times 5 mm dental blocks were prepared by sectioning the proximal surfaces of the teeth at 1 mm

apical to the cemento-enamel junction. The samples were divided into 5 groups; each photosensitizer was activated based on its standard wavelength as follows:

1.SRP alone (control): Fifteen dental blocks were only treated by SRP.

2.SRP + citric acid: After SRP, 15 dental blocks were treated with citric acid (pH=1) rubbed for 1 minute.

3.SRP + TB: After SRP, 15 dental blocks were treated with 0.1 mg/mL TB (Sigma, Germany) for 5 minutes in the dark at 37°C and were then irradiated with 635 nm diode laser (Konfleck, Taiwan) with an output power of 220 mW in continuous wave mode for 30 s from 1 cm distance with 17.18 J/cm² energy density. The diameter of laser hand-piece tip was 7 mm.

4.SRP+ MB: After SRP, 15 dental blocks were treated with MB (Sigma, Germany) and were then irradiated with diode laser with 660 nm wavelength (Konfleck, Taiwan), 150 mW output power, and 11.8 J/cm² energy density for 30 s.

5.SRP+ ICG: After SRP, 15 dental blocks were treated with ICG (Santa cruise, USA) and were then irradiated with 810 nm diode laser (Konfleck, Taiwan) with 250 mW output power and 19.5 J/cm² energy for 30 s.

The HGFs were purchased from the Pasteur Institute Cell Bank (Tehran, Iran). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Before cell culture, samples were sterilized by immersion in 70% ethanol for 24 hours. To assess the probability of microbial contamination, the samples were incubated in whole medium for 24 hours in 24-well culture plates and were examined under a light microscope. For cell culture experiments, HGFs were detached from the culture flask by trypsin and seeded on the surface of samples with a density of 10⁴ cells/sample. The plates were incubated at 37°C with 5% CO₂ for 72 hours.

Cell attachment was assessed by the methyl thiazolyl tetrazolium (MTT) assay after 24 hours. HGFs were treated with MTT reagent for 4 hours at 37°C (10% v/v of medium). The MTT assay measures the mitochondrial activity of the cells by assessing the conversion of water-soluble MTT to insoluble formazan. It is then

dissolved in 100 μ L of dimethyl sulfoxide to determine its optical density using an ELISA Reader at 570 nm wavelength (Anthos 2020, Austria) (13). Cell proliferation was also measured by the MTT assay at 48 and 72 hours using the above-mentioned protocol.

Statistical analysis:

Data were analyzed using SPSS version 22 (SPSS Inc., IL, USA). The normal distribution of data was evaluated using the Kolmogorov-Smirnov test. Group comparisons were done using repeated measures ANOVA, and pairwise comparisons of the time points were performed by the LSD test.

Results

The Kolmogorov-Smirnov test showed that the data were normally distributed ($P \geq 0.05$). Table 1 shows the fibroblastic adhesion (24 hours) and proliferation (48 and 72 hours) in the five groups.

Cell proliferation was higher in all experimental groups at 48 and 72 hours compared with 24 hours ($P < 0.05$). However, the experimental groups were not significantly different in terms of proliferation and adhesion of HGFs ($P = 0.143$). But, the observed values for PDT with ICG at 24 and 48 hours and the values for treatment with citric acid at 72 hours were slightly higher than the values in other groups. The type of treatment and time did not affect cell proliferation or adhesion ($P = 0.704$). Cell proliferation showed a significant difference at 72 hours compared with 48 hours ($P = 0.0001$).

Discussion

The results of the current study indicated that PDT and citric acid treatment as an adjunct to periodontal therapy did not have any effects on the adhesion or proliferation of HGFs according to the MTT assay. Although the proliferation of fibroblasts increased from 48 hours to 72 hours, this process progressed at the same rate in all groups, and was similar to SRP treatment alone. Fibroblasts have a spherical structure before exposure to the culture medium and do not have cytoplasmic processes. After incubation, they become close to the surface (dental blocks in this study) because of the gravity. If the

surface has the potential for fibroblastic adhesion, it would result in formation of weak Van der Waals bonds. Fibroblast receptors, called integrins, attach to tooth surface adhesins and form a stronger bond, and then cytoplasmic processes form a chemomechanical bond to the surface.

This in vitro study was not conducted to evaluate the antimicrobial activity of PDT, since this has been proven in previous studies (14-16). It aimed to evaluate the possible positive effects of various photosensitizers on HGFs.

After periodontal surgery, gingival fibroblasts are the first cells that adhere to the root surfaces; however, biological modifications may affect the adhesion of collagen fibers to root surfaces (1,17). Chemical treatments may increase the stability of the blood clot in the early stages of periodontal healing and enhance the adhesion of blood cells and fibrin (18).

Root conditioning with citric acid has shown beneficial results in cell culture (19,20) and animal (21) and human (22) studies. In the present study, treatment with citric acid increased fibroblast stimulation compared with the control group for up to 48 hours. Although the cell proliferation values at 72 hours were greater in the control group followed by the MB, TB, ICG and citric acid groups, there was no significant difference among the groups. The following two reasons may explain this finding: Firstly, residual chemical agents such as citric acid or photosensitizers may interfere with cell growth and proliferation, and secondly, there is a high possibility that the cells in all groups reached confluence within 72 hours. The latter possibility was also mentioned in another study (23). Feist et al, (24) also noted that fibroblasts reached confluence 3 days after culturing. Use of PDT in this study was because of the findings of previous studies (23).

Damante et al. (8) showed that TB solution (100 μ g/mL) significantly decreased the microhardness and roughness of dentin blocks by $7.9 \pm 7.9 \mu$ m, which was comparable to treatment with citric acid in order to stimulate the growth and proliferation of cells. Another study showed that the activity of keratinocytes

Table 1. Fibroblastic adhesion (24 hours) and proliferation (48 and 72 hours) by the MTT assay in the groups

Time point	Treatment	Mean \pm std. deviation	Minimum	Maximum
24 h	SRP	0.294 \pm 0.04	0.238	0.345
	SRP + CA	0.392 \pm 0.51	0.339	0.425
	SRP+MB	0.244 \pm 0.47	0.167	0.291
	SRP+TB	0.351 \pm 0.04	0.297	0.344
	SRP+ICG	0.384 \pm 0.11	0.222	0.532
48 h	SRP	0.257 \pm 0.30	0.208	0.293
	SRP + CA	0.513 \pm 0.18	0.369	0.667
	SRP+MB	0.311 \pm 0.05	0.306	0.320
	SRP+TB	0.380 \pm 0.10	0.249	0.529
	SRP+ICG	0.440 \pm 0.13	0.345	0.668
72 h	SRP	0.452 \pm 0.50	0.371	0.508
	SRP + CA	0.572 \pm 0.10	0.443	0.701
	SRP+MB	0.529 \pm 0.06	0.468	0.629
	SRP+TB	0.528 \pm 0.12	0.358	0.652
	SRP+ICG	0.502 \pm 0.06	0.436	0.590

SRP= Scaling and root planing, CA= Citric acid, MB= Methylene blue

TB= Toluidine blue, ICG= Indocyanine green

and fibroblasts decreased by 12% and 8%, respectively following the use of low concentrations of TB (25). However, in the present study, consistent with that of Karam et al, (23) and Damante et al, (8) PDT increased the fibrous stability by up to 72 hours. Moreover, there was no significant difference with citric acid at all time points. The reason for this difference in the findings is that in the study by Soukos et al, (25) TB was used in a culture medium that could cause cellular impairment, while in the clinical setting, the buffering capacity of tissues and gingival crevicular fluid may quickly diminish or even neutralize the effects of TB (25). The findings of the present study also confirmed the previous findings

regarding the toxicity of TB and possibly other photosensitizers (MB and ICG) for the bacteria and their safety for cells (26). In addition, histological analysis has not shown any tissue inflammation following TB exposure (27).

The present study was the first to compare the effects of PDT with TB, MB and ICG on proliferation and adhesion of HGFs to the human dental root surfaces in vitro. In presence of all three agents, cell adhesion and proliferation occurred at the same rate before treatment and increased at 24, 48 and 72 hours; thus, all three substances were safe for cell proliferation. Sperandio et al. (28) showed that PDT with MB had no effect on wound healing, fibroblasts or epithelial cells after surgery,

although laser irradiation alone had beneficial effects on accelerating the recovery process. Pourhajibagher et al. (29) showed that ICG significantly decreased the viability of HGFs at concentrations below 1000 µg/mL. They also noted that the rate of cytotoxicity was higher when the cells were irradiated twice (two cycles of 30 s each) with a 1-minute interval. It is worth noting that in our study, cell growth and proliferation was evaluated on hard surfaces compared with the culture media and the samples were exposed to the light source only once. Margaron et al. (27) reported that PDT inhibited cell adhesion and affected integrin signaling without modifying the cell membrane integrity or integrin expression.

Studies on the use of PDT in treatment of periodontal disease have shown different results so far, and they mainly focused on the antimicrobial properties of this therapeutic modality (15,16,25,29). Clinical evaluation, histological analysis, electron microscopy assessment and animal studies are required to further elucidate this topic.

Monzavi et al. (30) showed that aPDT with ICG as an adjunct to SRP yielded complete resolution of inflammation and reduction in pocket depth, but compared with SRP, it had no additional advantage in clinical attachment gain and plaque score. In a systematic review, Moslemi et al. (31) concluded that multiple dose is more effective in aPDT to achieve more appropriate results. Considering the results of these studies (30,31) and other similar studies (32-34), we might have obtained superior results if the number of applications had been doubled, but this requires further investigations.

Based on the results of this preliminary study on the effects of PDT on fibroblasts, it is concluded that PDT with TB, MB and ICG photosensitizers and treatment of dentin blocks with citric acid do not harm the growth and proliferation of HGFs.

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