Comparative effects of chlorhexidine and essential oils containing mouth rinse on stem cells cultured on a titanium surface

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Abstract. Chlorhexidine (CHX) and Listerine (LIS), an essential oil compound, are the two commonly used adjunctive agents for mechanical debridement, for reducing the bacterial load in the treatment of peri-implant inflammation. However, antimicrobial agents have been reported to be cytotoxic to the alveolar bone cells and gingival epithelial cells. The present study was performed to examine the effects of antiseptics CHX and LIS, on the morphology and proliferation of stem cells. Stem cells derived from the buccal fat pad were grown on machined titanium discs. Each disc was immersed in CHX or LIS for 30 sec, 1.5 min or 4.5 min. Cell morphology was evaluated with a confocal laser microscope and the viability of the cells was quantitatively analyzed with the cell counting kit-8 (CCK-8). The untreated cells attached to the titanium discs demonstrated well-organized actin cytoskeletons. No marked alterations in the cytoskeletal organization were observed in any of the treated groups. The treatment with CHX and LIS of the titanium discs decreased the viability of the cells grown on the treated discs ($P<0.05$). The stem cells derived from the buccal fat pad were sensitive to CHX and LIS, and a reduction in cellular viability was observed when these agents were applied to the discs for 30 sec. Further studies are required to determine the optimal application time and concentration of this antimicrobial agent for maximizing the reduction of the bacterial load and minimizing the cytotoxicity to the surrounding cells.

Introduction

Chlorhexidine (CHX), a cationic bisbiguanide with a broad antimicrobial spectrum, attacks the bacterial cell membrane, causing leakage and precipitation of the cellular contents (1). CHX is widely used clinically to reduce inflammation, and swelling and bleeding of the gums. As a result, it is currently recognized as one of the most effective chemical antiplaque agents (2,3). Listerine (LIS) is an essential oil agent, containing menthol, thymol, methyl salicylate and eucalyptol as active agents, which exerts a lethal effect on microbiota by disrupting the cell wall and inhibiting enzymatic activity (1,4).

When an implant surface is exposed to the oral cavity, it is immediately covered by the salivary pellicle and colonized by oral microorganisms (5,6). Mechanical instrumentation with metal curettes, plastic curettes, ultrasonic scalers, air-powder abrasive systems and lasers, has been widely used to remove plaque from dental implants (7,8). However, it has been demonstrated that it is impossible to achieve complete removal of all of the adhering microorganisms by mechanical debridement, due to the complexity of the implant surfaces provided with threads or roughness (9,10). Therefore, adjunctive peri-implant therapies, including antibiotics and antiseptics, have been proposed (11). CHX and LIS are used as alternative or adjunctive treatments to mechanical debridement for reducing the bacterial load in the peri-implant pocket (5,12). CHX and LIS are reported to inhibit biofilm formation in an *in vitro* model (12,13). One possible drawback to CHX use is that CHX has been demonstrated to exhibit cytotoxic activity on alveolar bone cells and gingival epithelial cells (14,15).

Previous studies have reported that mesenchymal stem cells served as initial colonizers of implant surfaces (16,17). The buccal fat pad is an readily accessible source of adult stem cells (18). The present study aimed to investigate the effects of the two antiseptics, CHX and LIS, on the buccal fat pad derived stem cells grown on titanium discs.

Materials and methods

Tissue preparation and cell isolation/expansion. The buccal fat pad was obtained from a healthy individual undergoing orthognathic surgery procedures in the Seoul St. Mary's Hospital (Seoul, Korea). The patient was in good health and no systemic diseases were reported. This study was reviewed and approved by the Institutional Review Board of the Catholic University of Korea (Seoul, Korea) and informed consent was

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obtained from the patient. Sample tissues were processed according to a previously described method (19,20). The tissues were washed several times with sterile phosphate-buffered saline (PBS; Invitrogen Life Technologies, Carlsbad, CA, USA), ground into small pieces and treated with 0.06 % collagenase I (Invitrogen Life Technologies) for 4 h at 37˚C. Following incubation, the tissue was centrifuged at 100 x g for 10 min to separate the adipocytes and lipid droplets from the stromal vascular fraction. Then, the cells were passed through a 40 μm cell strainer (BD Biosciences, Bedford, MA, USA). The cells were then re-suspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies), containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Logan, UT, USA) and antibiotics (100 U/ml of penicillin and 100 μg/ml streptomycin; Invitrogen Life Technologies). The cells were seeded in 100 mm tissue culture dishes and maintained at 37˚C in a humidified 5% CO_2 environment. The culture media were replenished with fresh media every two days. The cells were sub-cultured with 0.05% trypsin-EDTA (Invitrogen Life Technologies) at 5- to 7-day intervals at a 1:2-4 dilution. The initial adherent cell population, referred to as passage 0 (P0), as well as several of the following passages (up to three passages), were analyzed by flow cytometry (BD Biosciences).

Cell culture on titanium discs and antiseptic application. Fig. 1 illustrates the overview of the study design. Machined titanium discs (15) measuring 10 mm in diameter and 2 mm in thickness were used in this study (Dentium Co., Seoul, Korea). Two mouth rinses were applied; (i) a 0.12 % chlorhexidine digluconate solution (CHX; Hexamedine, Bukwang, Seoul, Korea) and (ii) a solution containing essential oils (LIS, Listerine® Coolmint; Johnson & Johnson, Bangkok, Thailand). The stem cells were plated at a density of $3.0x10^4$ cells/well on 24-well plates containing titanium discs and cultured in DMEM for nine days. The media in each were suctioned away and the discs were immersed either in CHX or LIS for 30 sec, 1.5 min or 4.5 min.

Cell viability test. The viability of the treated cells was quantitatively analyzed by a cell counting kit-8 (CCK-8; Dojindo Molecular Technologies Inc., Rockville, MD, USA). A water-soluble tetrazolium salt-8 [2-(2-methoxy- 4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo phenyl)-2H-tetrazolium, monosodium salt (WST-8)] solution was added to the wells and the discs were incubated for 3 h. The quantity of generated formazan at the 1 and 3 h incubation time points was determined by reading the absorbance at a 450 nm wavelength using the microplate spectrophotometer system (BioTek, Winooski, VT, USA) (21).

Evaluation of cell morphology. Following the cell viability test, each implant disc was fixed with 4% paraformaldehyde overnight. The discs were washed in PBS three times. Actin filaments were stained with rhodamine-conjugated-phalloidin (Molecular Probes, Eugene, OR, USA) and the nuclei were counterstained with Hoechst 33342 blue dye (Molecular probes). The cells were observed using a confocal laser microscope (LSM5 Pascal; Carl Zeiss AG, Jena, Germany) at a magnification of x400.

Statistical analysis. The results were represented as the mean ± standard deviation. An analysis of covariance (ANCOVA) was performed to compare cellular viability, according to group and time, using commercially available statistical software (SPSS 12 for Windows; SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Evaluation of cell morphology. The untreated cells attached to the titanium discs demonstrated well-organized actin cytoskeletons with blue nuclei under a confocal microscope (Fig. 2). The treatment of the adult stem cells with 0.12% CHX for 30 sec did not cause a significant alteration compared with the untreated group. Increasing the immersion times (1.5 and 4.5 min) did not lead to significant changes. A similar trend was observed in the LIS groups. No notable alteration in the cytoskeletal organization was observed. Rounding of the cells or progressive detachment from the substrate was not observed during the experiments.

Cellular viability. An CCK-8 assay demonstrated that the treatment with CHX and LIS affected cell viability. The CHX and LIS demonstrated toxic effects on adult stem cells *in vitro*, with a mean viability of 84.8±0.4 and 81.3±1.5%, respectively, following exposure for 30 sec, when the control group was considered to be 100% (100±2.1; $P<0.05$; Fig. 3). The increase in the treatment time of CHX and LIS up to 4.5 min did not induce significant decreases of viability. Mean cell viability for the CHX group was 84.2±1.1 and 84.8±0.4% for 1.5 and 4.5 min, and the viability of the LIS group for 1.5 and 4.5 min was 82.1±1.9 and 82.1±0.4%, respectively. The progressive increase in the treatment time up to 4.5 min did not induce any additional decreases of viability either in CHX and LIS at 1 h ($P>0.05$).

The relative viability of CHX of 30 sec, 1.5 and 4.5 min at 3 h was 74.6±0.3, 74.8±0.6 and 73.3±0.6, respectively, when the control group was considered 100% (100±1.5; Fig. 4). The relative viability of LIS of 30 sec, 1.5 and 4.5 min was 71.8±0.9, 73.7±1.2 and 73.3±1.2%, respectively. The 3 h results were significantly lesser than the data from 1 h ($P<0.05$).

Discussion

The present study clearly demonstrated that stem cells derived from the buccal fat pad were sensitive to CHX and LIS, and that these cells experienced a decrease in cellular viability with the application time of 30 sec.

A previous study demonstrated that CHX was more cytotoxic than LIS for cultured human gingival fibroblasts, however LIS appeared to be more cytotoxic than CHX at a diluted concentration (1 and 2% of the given solutions) (22). In the present study, for the stem cells from the buccal fat pad, there was no significant difference of viability between CHX and LIS. Previous studies have identified that CHX induced cell damage in a time-dependent manner for osteoblastic cells and fibroblasts (23). However, this study did not demonstrated significant decreases of viability from 30 sec to 4.5 min in application. The conflicting results regarding the different

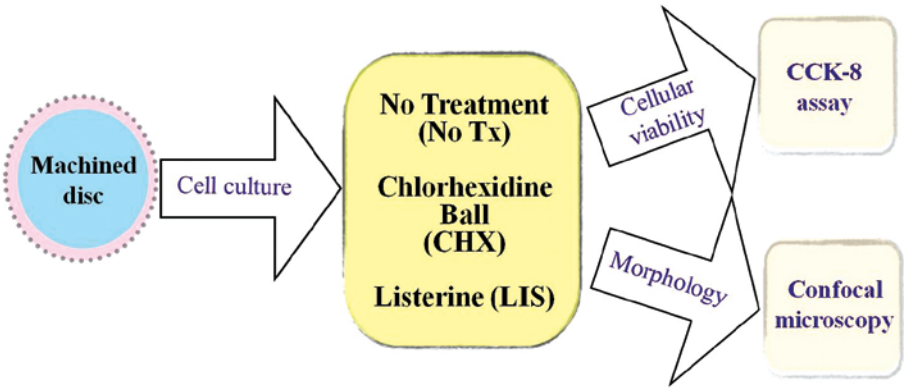

Figure 1. A schematic diagram of the overview of the study design. CCK-8, cell counting kit-8; Tx, treatment; CHX, chlorhexidine; LIS, Listerine.

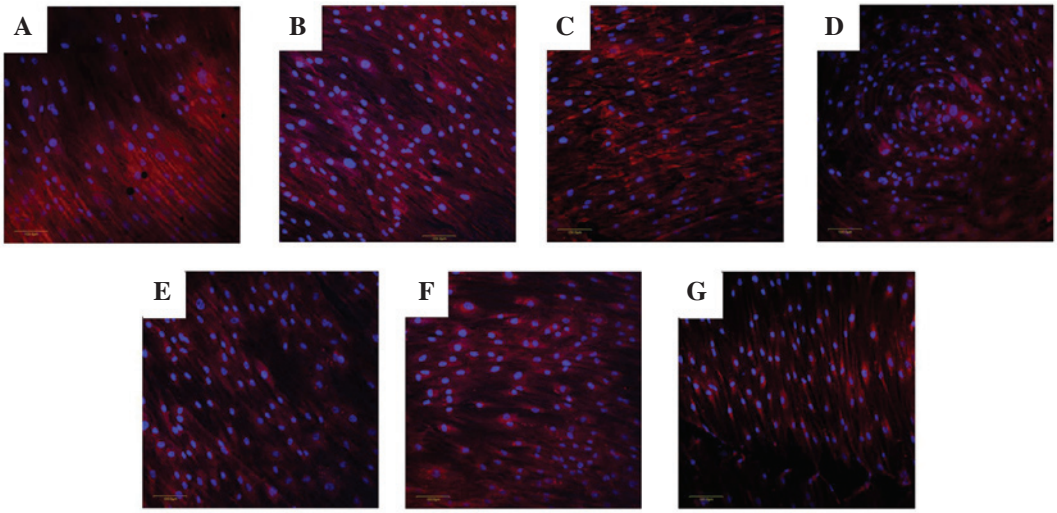

Figure 2. Evaluation of cell morphology. (A) Control; (B) CHX for 30 sec; (C) CHX for 1.5 min; (D) CHX for 4.5 min; (E) LIS for 30 sec; (F) LIS for 1.5 min; (G) LIS for 4.5 min. CHX, chlorhexidine; LIS, Listerine.

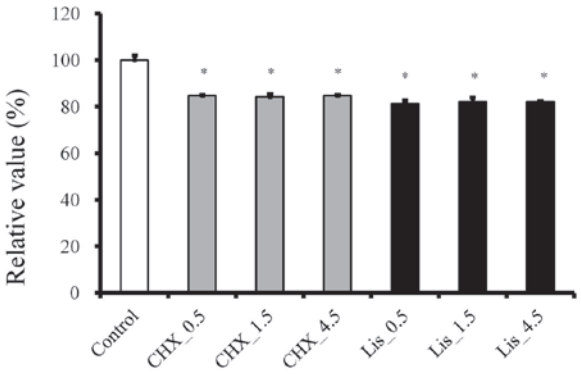

Figure 3. Cellular viability at 1 h. CHX, chlorhexidine; LIS, Listerine.

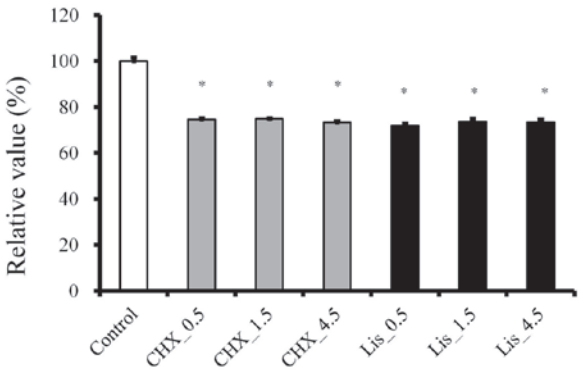

Figure 4. Relative viability at 3 h. CHX, chlorhexidine; LIS, Listerine.

responses to CHX and LIS may, in part, be attributed to the type of cells, culturing period, stage of differentiation of the cells or culturing condition (24).

The data describing the effects of CHX and LIS are varied between different studies (5,13,25). It was demonstrated that CHX and LIS were able to reduce the total amount of microorganisms accumulating on titanium surfaces and that they exhibited a significant bactericidal effect against adhering bacteria (5). However, a significant reduction in the amount of bacteria in the saliva was observed after the chlorhexidine mouth rinse, but not following the essential oils rinse (25). In one study, CHX and LIS did not demonstrate a

broad-spectrum antimicrobial effect, so they were not recommended for the detoxification of infected implant surfaces (13). Further investigations are required to determine the optimal application time and concentration of the antimicrobial agent to maximize the reduction of the bacterial load and minimize the cytotoxicity to the surrounding cells.

CHX has the property of substantivity, allowing prolonged adherence of the antiseptic to hard and soft oral surfaces and its gradual release at effective doses produces the persistence of its antimicrobial activity (26). Higher antibacterial effects of CHX were observed in the rough titanium surface when compared with the machined surface (3) and more pronounced effects of CHX may be observed if the stem cells were applied onto the rough surface.

It has been demonstrated that bacterial growth inhibition is affected by the concentration of the antimicrobial agent (3,27). The effects of rinsing of 0.12% CHX was compared with irrigation with 0.06% CHX using a powered oral irrigator and the results revealed that the irrigation group also demonstrated a greater reduction in the bleeding index and the calculus index than the rinsing group (28). This approach, using the powered irrigator with a diluted solution, may be considered as an adjunct to oral health in patients with implants and may produce less cytotoxic effects to the surrounding cells.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is considered to be a more sensitive assay than the trypan blue assay (29). Trypan blue assay is based on the principle that live cells possess intact cell membranes that exclude penetration of the dye, while the MTT assay assesses cellular viability through the determination of mitochondrial dehydrogenase activity. However, further treatment is required to solubilize the formazan crystals and MTT may be toxic to cells (30). In the present study, a CCK-8 assay utilizing WST-8 was used because this is reported to be more sensitive than the MTT assay and less toxic to the cells (30).

From these results, we conclude that the application of CHX and LIS on titanium discs had residual effects on the viability of the stem cells derived from the buccal fat pad, and that it may be suggested that the application of CHX or LIS directly into the peri-implant pocket produces toxic effects. The concentration and application time of the antimicrobial agent should be meticulously controlled to obtain optimal results. These cytotoxic effects should be considered if regenerative surgery is planned for the treatment of peri-implantitis.

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