Clinical implications of the growth-suppressive effects of chlorhexidine at low and high concentrations on human gingival fibroblasts and changes in morphology

MARZENA WYGANOWSKA-SWIATKOWSKA[1], MALGORZATA KOTWICKA[2], PAULINA URBANIAK[2], AGNIESZKA NOWAK[3], EWA SKRZYPCZAK-JANKUN[4] and JERZY JANKUN[4]

[1]Department of Conservative Dentistry and Periodontology, Poznan University of Medical Sciences, 60-812 Poznan; [2]Department of Cell Biology, Poznan University of Medical Sciences, 60-806 Poznan; [3]Department of Biochemistry and Biotechnology, Poznan University of Life Sciences, 60-632 Poznan, Poland; [4]Urology Research Center, Department of Urology, College of Medicine, University of Toledo, Toledo, OH 43614, USA

Abstract. Chlorhexidine (CHX) is considered the gold standard in the antiseptic treatment of the oral cavity, due to its high antibactericidal capability. With the use of CHX mouth-rinse formulations, the bacteriostatic effects are maintained by the adsorption and prolonged release of CHX from oral surfaces. It was believed that antiplaque formation ability and the lack of systemic toxicity of CHX render it an excellent antiseptic in post-surgical dental treatment. However, recent studies have demonstrated that CHX exerts cytotoxic effects on human periodontal tissues, such as gingival fibroblasts and other cells. It also reduces gingival fibroblast adhesion to fibronectin and prevents fibroblast attachment to root surfaces, thus interfering with periodontal regeneration. In this study, using human gingival fibroblasts (HGFs), we investigated effects of CHX on the growth, morphology and proliferation of HGFs. We found that a low concentration (0.002%) of CHX does not interfere with the proliferation and morphology of HGFs. However, a higher concentration (≥0.04%) of CHX inhibits cell proliferation and to a certain extent, affects cell morphology in a time-dependent manner. A decrease in the percentage of cells in the G_0/G_1 phase and the accumulation of cells in the S phase following treatment with CHX also occurred in a dose-dependent manner. We thus concluded that CHX only at the concentration of 0.002% does not interfere with HGF growth, that is so critical to wound healing. Thus, the application of CHX in the post-surgical antiseptic treatment of the oral cavity should be limited.

Introduction

Chlorhexidine (CHX) is considered the gold standard in the antiseptic treatment of the oral cavity, due to its high antibactericidal capability (1,2), its inhibitory effects on glycosidic and proteolytic (3) and matrix metalloproteinase activities (4), and its reducing effecfts on the leucocyte concentration (to basal levels) and on pro-inflamatory cytokines (5). CHX is an antimicrobial agent that belongs to the group of N^5 derivatives of 1:6-bis-biguanidohexane (6,7) and is also effective in the treatment of non-bacterial oral infections. CHX binds to negatively charged sites on the bacterial surface wall through electrostatic forces. Such an interaction affects the membrane structure and causes the leakage of intracellular bacterial components (1,8,9).

With the use of CHX mouth-rinse formulations, there is an immediate bactericidal effect due to cytoplasmic precipitation. The bacteriostatic effect is further induced by the adsorption and prolonged release of CHX from oral surfaces (1,10). This antiplaque formation effect and lack of systemic toxicity (5) render CHX a commonly used antiseptic in post-surgical dental treatment. However, recent studies have demonstrated that CHX exerts potent cytotoxic effects on human periodontal tissues, such as gingival fibroblasts (11,12), gingival epithelial cells (13), periodontal ligament cells (14), cultured alveolar bone cell (15) and on osteoblastic cells (7). It also reduces gingival fibroblast adhesion to fibronectin (16) and prevents fibroblast attachment to root surfaces; thus, it can interfere with periodontal treatment and regeneration (7). Yet it is difficult to compare all the published results, as they refer to the different commercial mouth rinsing fluids containing CHX, each one containing different concentrations of this active chemical agent. Some of these mouth rinsing fluids also contain alcohol, which can influence cell proliferation and morphology.

Our previous study indicated that an alcohol concentration of 10% does not inhibit fibroblast proliferation and the presence of alcohol in mouth rinsing fluids containing 0.10% CHX has no

Correspondence to: Dr Marzena Wyganowska-Swiatkowska, Department of Conservative Dentistry and Periodontology, Collegium Stomatologicum, Poznan University of Medical Sciences, 70 Bukowska Street, 60-812 Poznan, Poland
E-mail: marzena.wyganowska@periona.pl

Professor Jerzy Jankun, Urology Research Center, Department of Urology, College of Medicine, University of Toledo, 3000 Arlington Avenue, Toledo, OH 43614, USA
E-mail: jerzy.jankun@utoledo.edu

deleterious effects on healing capacity (17). On the contrary, it helps stimulate wound healing (11). In addition, the culture media used in *in vitro* experiments differ [fetal bovine serum (FBS) or calf bovine serum]. Usually, experiments for evaluating the cytotoxicity of antiseptics are carried out in cell culture medium containing 10% FBS, which is similar to the composition of artificial wound fluid (18); however, FBS has an attenuating effect against CHX-induced cytotoxicity (8). Although a number of studies have demonstrated the cytotoxicity of CHX (12,13,16), none of the observations lasted for >24 h and none of the studies used the short-cut video to demonstrate the results. Moreover, in this study, we used the PANsys3000 system to examine the effects of CHX on human gingival fibroblasts (HGFs) cultured without FBS. PANsys3000 is a highly automated cell-culture system that is used for *in vitro* cell culture and for the analysis of diverse cell lines in conditions similar to those observed *in vivo*. This system enables the culture of various cells and the usage of diverse culture media at the same time, using the cell culture conditions of choice and constant microscopic observation. Simultaneously, in our study, we applied the xCELLigance real-time cell analysis (RTCA) system as a non-invasive and label-free approach to assess cell proliferation in real-time on a cell culture level.

Materials and methods

Cell culture. All experiments were conducted using a human gingival fibroblast (HGF) cell line (reference no. P10866; Innoprot, Biscay, Spain). Gingival fibroblasts were transferred in aseptic conditions from freezing medium [Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1), 10% FBS, 10% dimethyl sulfoxide (DMSO) (all from Gibco, Grand Island, NY, USA)], to a 90-mm sterile petri dish (Sarstedt, Nuembrecht, Germany) containing 10 ml of growth medium with the following composition: DMEM/F12 (1:1) medium, 10% FBS, antibiotics (penicillin 100 μg/ml and streptomycin 100 μg/ml) and 2 mmol/l L-glutamine (all from Gibco). The cells were grown in aseptic conditions, in an incubator at 37˚C with 5% CO_2 and 100% humidity. The cells were cultured until 90% confluent. At this point, they were washed with phosphate-buffered saline (PBS) and trypsinized with trypsin/EDTA solution (0.25% trypsin containing 0.01% EDTA). After 5 min of incubation, complete growth medium was added, and the cell suspension was transferred to petri dishes.

Stimulation of gingival fibroblasts with CHX. To evaluate the effecs of CHX on fibroblasts, the cells were grown in regular culture medium for 24 h. The medium was then replaced with appropriate CHX dilutions. The practical dilution was obtained by dissolving commercially available CHX solution [Curasept ADS 220 (0.2% CHX)] in FBS-free medium. The final dilutions of CHX in the FBS-free medium were as follows: 0.002, 0.01, 0.02, 0.04 and 0.2%. The cells were stimulated with CHX for 15 min and the solutions were then replaced with regular growth medium and the cells were grown under standard conditions for 48 h.

Analysis of cell growth and morphology. Cell growth and morphology were assayed using PANsys3000. PANsys 3000 (Systech GmbH, Augsburg, Germany) is a multi-chamber fully automated cell culture system used for *in vitro* experiments simulating *in vivo* conditions. It allows the culture of different cell types and several components simultaneously with a variety of culture conditions and continuous microscopic observations. The parameter defined as the cell index (CI) represents cell growth, measuring the relative change in electrical impedance in the presence or absence of cells in the wells. CI is a unitless parameter and is calculated using the following formula: $CI = (Z_i-Z_0)/15$ where Z_i is the impedance during the experiment and Z_0 is the impedance at the beginning of the experiment (19-21).

The cells were grown prior to the experiment for 24 h in an incubator at 37˚C with 5% CO_2 and 90% humidity (ftp.strefa.pl, user: m.wyganowska+kdvision.eu; password: Wyga1, supplementary 1.avi). Subsequently, the growth media were removed and replaced with the appropriate CHX dilutions (0.002, 0.01, 0.02, 0.04 and 0.2%) and the cells were resuspended in 1 ml of DMEM FBS-free medium. The control cells were treated with 1 ml of DMEM FBS-free medium. The cells were incubated for 15 min at 37˚C. After the CHX solution was removed, the cells were rinsed with Hank's solution (Cytogen, Wetzlar, Germany) and complete growth medium was added. Further observations were conducted for the following 48 h. Images were acquired at 10-min intervals and finally combined into a video. All of the images were acquired in the same plate region (region of interest).

Assessment of cell proliferation rate. Real-time cell analyses (xCELLigence system; Roche Applied Science, Mannheim, Germany; ACEA Biosciences, San Diego, CA, USA) were performed to determine the effects of CHX on gingival fibroblast proliferation. The electronic impedance of the sensor electrodes was measured to allow the monitoring and detection of physiologic changes of the cells on the electrodes. The voltage applied to the electrodes during real-time cell analysis was approximately 20 mV root mean square. The impedance measured between electrodes in a well depends on electrode geometry, the ion concentration in the well, and whether the cells are attached to the electrodes. In the presence of cells, cells attached to the electrode sensor surfaces act as insulators, and thereby alter the local ion environment at the electrode-solution interface, leading to increased impedance. Thus, the larger the value of electrode impedance, the larger the number of cells growing on the electrodes.

During the cell proliferation measurements, the cells were passaged after reaching confluency and were trypsinized with 0.25% trypsin. After seeding 200 μl of the cell suspensions into the wells (10,000 cells/well) of the E-plate 96 (ACEA Biosciences), the HGFs were kept in culture to obtain the CI value of approximately 2. Subsequently, the cells were treated with the appropriate dilutions of CHX and released from the metallic alloy material of the electrodes and monitored every 15 min for 48 h. The control plate contained cells not stimulated with CHX, but with the replacement of the growth medium with FBS-free medium and were then cultured in complete culture medium.

Cell cycle analysis. The cells were seeded in 60-mm culture dishes at a density of 5x10^5 cells/dish and allowed to adhere overnight. Following 15 min of incubation with CHX at dilutions (0.002, 0.004, 0.01, 0.02, 0.04 and 0.2%), the cells were washed twice with PBS and the solutions were then replaced
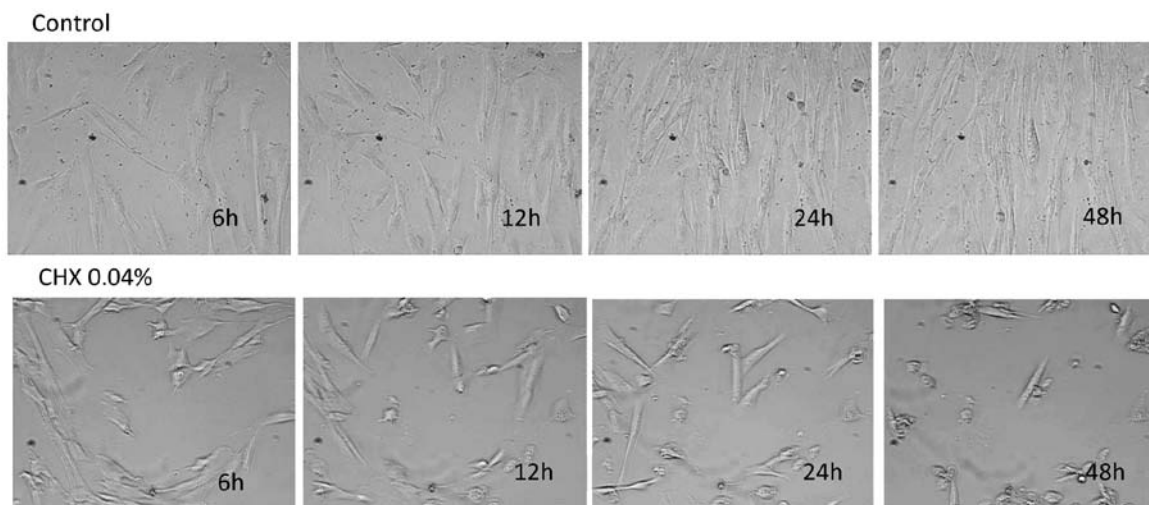
Figure 1. Representative images acquired using the PANsys3000 system showing the effects of chlorhexidine (CHX) on human gingival fibroblasts during 48 h of observation following stimulation. The control cells were treated with 1 ml of DMEM FBS-free medium. The fibroblasts treated with CHX at a concentration of 0.04% were small in number, and had an oval appearance with no filopodia.

with regular growth medium, and the cells were grown under standard conditions for 48 h. Subsequently, the cells were trypsinized (trypsin; Cytogen) and fixed with ice-cold 70% ethanol at -20°C for 24 h. Subsequently, the cells were centrifuged, washed once with PBS, and then incubated with RNAse A (50 µg/ml in PBS) for 30 min. Following centrifugation at 100 rpm for 10 min at 4°C, the supernatant with RNAse A was removed and intracellular DNA was labeled with 0.5 ml of cold propidium iodide (PI) solution (0.1% Triton X-100, 0.1 mM EDTA, 50 µg/ml PI in PBS) on ice for 30 min in the dark. Cell cycle distribution was measured using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). For each experiment, 10,000 cells were examined. The fluorescence of PI was excited using an argon laser (488 nm). The emission of red fluorescence of PI was detected in the FL3 channel (>650 nm) All data were collected and analyzed using CellQuest Pro software (v.5.2.1) (Becton-Dickinson, Franklin Lakes, NJ, USA). The distribution of cells in the cell cycle (G_0/G_1, S and G_2/M) and apoptosis were calculated using the ModFit LT program for cell cycle analysis (Verity Software House Inc., Topsham, ME, USA).

Statistical analysis. Statistical analysis was performed using Statistica v.10 (StatSoft, Inc., Tulsa, UK). The Shapiro-Wilk test was used for the normality test of continuous variables. The mean ± standard deviation was used to describe the results of the experiments. The parametric test one-way ANOVA with the multiple comparison Tukey's post-test were applied. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Cell growth and morphology. In the control group, fibroblast morphology did not vary significantly during the duration of the experiment. During the 48 h of culture after DMEM stimulation, the gingival fibroblasts formed a confluent layer with lamellipodia and spreading of the cellular matrix (Fig. 1, top panel) (ftp.strefa.pl, user: m.wyganowska+kdvision.eu; password: Wyga1, supplementary 2.avi). Morphologically, no significant difference was

observed between the control cells and the CHX 0.002%-stimulated cells. Both groups exhibited a characteristic spindle-shaped fibroblast morphology. In the cells stimulated with CHX at the concentration of 0.01 and 0.02%, a decrease in cell proliferation and a decrease in the number of cell divisions were noted (ftp.strefa.pl, user: m.wyganowska+kdvision.eu; password: Wyga1, supplementary 3.avi). There were no significant changes observed in the morphology of the fibroblasts between both groups. In the cells stimulated with CHX at the concentration of either 0.04 or 0.2%, a progressive inhibition of cell growth and division was observed (ftp.strefa.pl, user: m.wyganowska+kdvision.eu; password: Wyga1, supplementary 4.avi). The growth inhibition was accompanied by the appearance of the small round-shaped cells (Fig. 1, bottom panel).

Cell proliferation rate. Cell proliferation assays were performed using the xCELLigence system. After seeding the HGFs into the wells, the mean impedance change (n=5) was measured. Impedance was recorded every 15 min. To improve the clarity of the presentation of the results, only 4 post-CHX stimulation read-outs were analyzed: at 12, 24, 36 and 48 h. No stimulated HGFs obtained a CI value of approximately 2 after 24 h of culture (Fig. 2). The control cells (treated with DMEM FBS-free medium) exhibited a significant increase in the cell index, which at 12 h after stimulation attained a value of 1.5±0.5 and increased to 2.6±0.6 after 48 h of incubation (p=0.003). The anti-proliferative concentration- and time-dependent effects of CHX on the HGFs are shown in Fig. 3. The cells stimulated with CHX at the concentration of 0.002% exhibited a significant increase in the CI value at 48 h (p<0.05), albeit significantly lower (p<0.05) than the control group. At higher CHX concentrations, the effects were less pronounced at the concentration of 0.01%, almost leveled off at the concentration of 0.02%, and were reversed at the concentrations of 0.04 and 0.2%, with insignificant increase in CI values (p>0.05) at the concentrations of 0.02 and 0.04% observed at 24 h as shown in Fig. 3.

Cell cycle analysis. Flow cytometry was used to examine the changes in the cell cycle of the HGFs that were either not stimu-
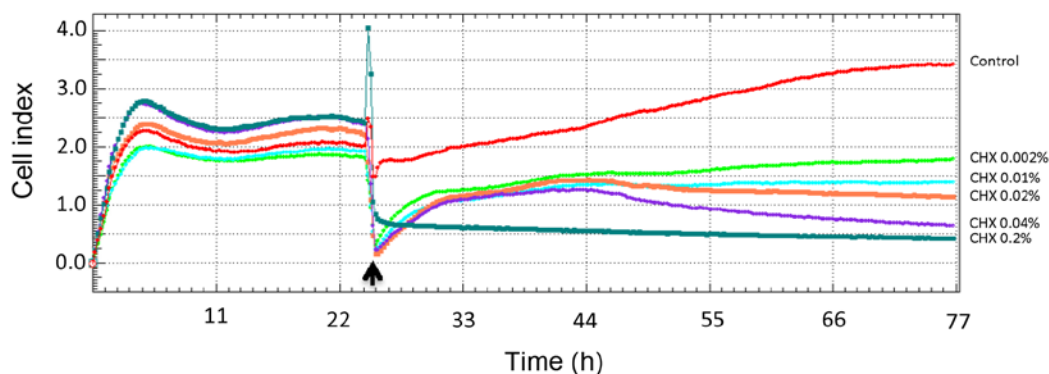
Figure 2. Real-time, label-free monitoring of the effects of chlorhexidine (CHX) on human gingival fibroblat proliferation using the xCELLigance system. A representative graph is shown. Human gingival fibroblasts were treated with various concentrations of CHX. The cell index value was monitored continuously for 48 h following stimulation with CHX (black arrow indicates the time of CHX administration).
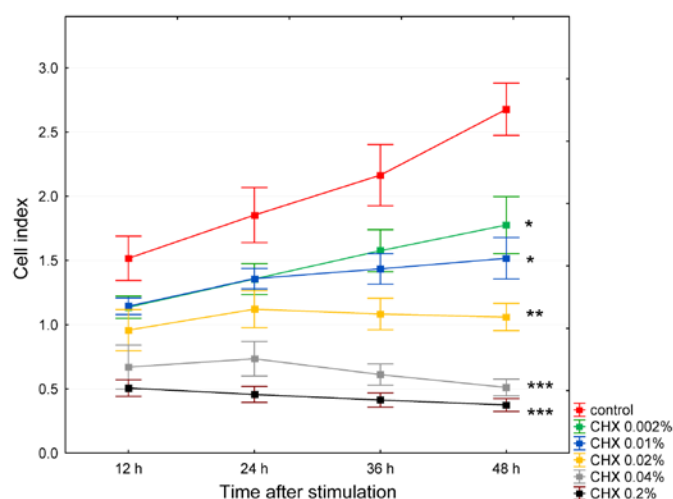

Figure 3. Concentration- and time-dependent inhibition of human gingival fibroblast proliferation following treatment with chlorhexidine at various concentrations; the results are presented as the means ± standard deviation. *P<0.05, **P<.001 and ***P<0.0001.

lated, or stimulated with CHX. The separation of the cells into apoptotic and the G_0/G_1, S or G_2/M phases was based upon linear fluorescence intensity after staining with PI. Representative profiles are shown in Fig. 4A. A decrease in the percentage of cells in the G_0/G_1 phase and a buildup of cells in the S phase was observed. This process was concentration-dependent. The HGFs not stimulated with CHX had 91.5±2.5% of cells in the G_0/G_1 phase, whereas the cells stimulated with 0.002, 0.01, 0.02, 0.04 or 0.2% CHX had 88.0±3.7, 85.4±2.4, 80.9±1.1, 79.4±5.7 and 65.1±2.7% of cells in the G_0/G_1 phase, respectively (Fig. 4B). In the control group, the percentage of cells in the S phase was 2.8±0.7%. Following stimulation with CHX, a concentration-dependent increase in the percentage of cells in the S phase was observed; the cells stimulated with the highest concentration of CHX had 22.1±1.3% of cells in the S phase (Fig. 4B). No apoptosis was observed either in the unstimulated or in the cells stimulated with 0.002 or 0.01% CHX. With the increasing CHX concentration, a significant enhancement in the percentage of cells undergoing apoptosis was detected, with the highest concentration corresponding to 9.0±2.6% of apoptotic cells (Fig. 4B).

Discussion

CHX has been widely utilized as a wound antiseptic and oral antimicrobial rinse. There have been numerous reports on its safety as an oral rinse; however, its effects on wound healing have been contradictory. It has been suggested that the direct application of CHX during regenerative periodontal therapy could have severe toxic effects on gingival fibroblasts, endothelial cells and alveolar osteoblasts, thus negatively interfering with the early healing phase (7).

In a previous study using an infected animal wound healing model with the polymer drug delivery system of PDGF, the use of hydrophilic protein promoting healing and CHX, a hydrophobic antimicrobial agent, effectively inhibited the proliferation of bacteria without exhibiting cytotoxicity to mammalian cells (22). However, CHX has been shown to induce an inflammatory reaction (23), tissue necrosis (24), and to retard the granulation of tissue formation and wound healing (25). Some other studies have established that CHX inhibits cell growth, proliferation and collagen synthesis in human osteoblasts (7,26) and human alveolar bone cells (15).

The comparison of the results from studies on the effects of CHX on periodontal tissue is complicated and practically impossible due to the different research methodologies applied by different authors; in particular, the duration of cell exposure, the CHX concentrations and the media used. Therefore, the most important aim of this study was to use the methods (PANsys 3000, xCELLigence) that allow us to observe the effects of CHX on cell lines in conditions similar to those observed *in vivo* and in real-time, and to deliver the most reliable results.

For this purpose, all experiments were conducted without medium containing fetal bovine, which is usually used for the similarity to the artificial wound fluid. It has been indicated that FBS has an attenuating effect against CHX-induced cytotoxicity, which results in a higher cell survival rate (8).

There are a number of different suggestions in the literature (1,7,24,27) for the duration of cell exposure to CHX during *in vitro* experiments. Due to the slow release of CHX from the tooth surface and soft tissue following application, it maintains its antimicrobial activity in the oral cavity for extended periods. During this time, the oral tissues are exposed to progressively lower concentrations of CHX. Furthermore, the periodontal pocket is a specific environment
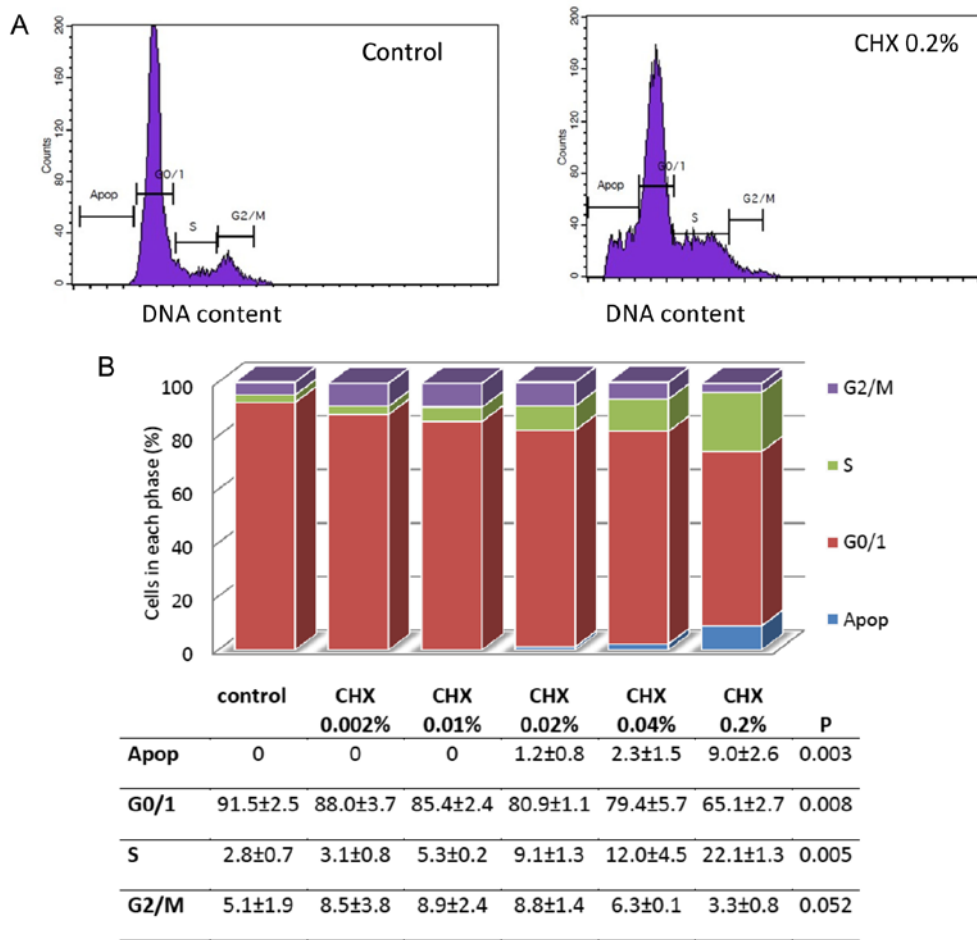
Figure 4. Flow cytometry-based cell cycle analysis of human gingival fibroblasts treated with chlorhexidine. (A) Representative histogram of DNA content for controls vs. chlorhexidine (CHX; 0.2%)-stimulated cells. (B) A significant decline in the percentage of G_1 and G_2/M cells was observed, along with a considerable augmentation in the percentage of apoptotic and S phase cells.

in which the gingival crevicular fluid is replaced approximately 40 times/h (28) and is usually penetrated by mouth rinse only to approximately 4% of its depth during mouth rinsing. Therefore, we decided to expose gingival fibroblasts to a CHX dilution for longer periods of time than the standard time of oral rinsing, but shorter than the expected release time from soft tissue.

In this study, during the constant microscopic observation of cell morphology and growth in conditions similar to those observed *in vivo*, we observed that either cell morphology or growth did not exhibit any changes in comparison with the control group following stimulation with 0.002% CHX. In the cells stimulated with CHX at the concentrations of 0.01 and 0.02% a decrease in both the dynamics of cell proliferation and the number of cell divisions was noted, although only in the final hours of observation. There were no significant changes observed in fibroblast morphology between these two groups. In the cells stimulated with 0.04 or 0.2% CHX, the progressive inhibition of growth and cell division was observed, which was most significant at 32 h with CHX at 0.04%, and at 16 h with CHX at 0.2%. At these time points, only small round-shaped cells were observed. These results are different from the ones presented in the literature. Giannelli *et al* (7) used the concentration of CHX similar to ours; however, following long-term treatment (5 and 15 min), the authors observed massive

cell death with any concentration used (using the calorimetric method and confocal microscopy). The results were established 4 h after exposure. Even following short-term treatment (1 min) with higher concentrations of CHX (0.03-0.12%) a significant reduction in cell viability was observed.

The only comparable results of fibroblast morphology were achieved for fibroblast stimulated with 0.002% CHX, even if the time of exposure differed. In our study it was after 15 min of treatment, after 1 min of treatment in the study by Giannelli *et al* (7), 24 h in the study by Dogan *et al* (1) and 1 h in the study by Pucher and Daniel (27). Faria *et al* (25), using CHX at the concentration of 0.001% observed many morphological changes in the fibroblasts, and the cells were completely destroyed from the concentration of 0.004%. These effects were observed using a scanning electron microscope directly following short-term stimulation. The discrepancies between these results may be due to the heterogeneity of human and animal (murine) fibroblasts, and the different investigation methods used in the different studies (1,27).

On the other hand, in the study by Ros-Llor and Lopez-Jornet (29), they did not report any genotoxic effects against oral mucosa cells resulting from mouth rinse containing CHX. The study evaluated DNA damage, cytokinetic defects, proliferative potential and cell death caused by the frequent use of triclosan, CHX and essential oils in ethanol solutions. No

nuclear abnormalities in exfoliated cells, collected from cheeks with a cytobrush, were observed (29).

Our data concur with those obtained by other authors (1,12,30), and confirmed the anti-proliferative effects of CHX on HGFs in *in vitro* conditions. The *in vitro* cytotoxicity of CHX occurred in a concentration and time-dependent manner. Moreover, Mariotti and Rumpf (12) postulated that CHX, in concentrations which have little effect on cellular proliferation, can significantly reduce both collagen and non-collagen protein production by HGFs *in vitro*.

In this study, we used the RTCA technique (xCELLigance RTCA system) to provide real-time data concerning the way that CHX alters the behavior of fibroblasts. The xCELLigance RTCA platform is highly accurate for monitoring cell behavior and it correlates very well with conventional adhesion, proliferation and migration assays. This non-invasive and label-free platform is being used as a robust system to measure the toxicological response to nanoparticles and novel compounds (31,32). Having exposed the HGFs to CHX, we were able to demonstrate a significant decrease in CI, which correlated with a decrease in cell proliferation.

Due to the fact that the xCELLigance system is an impedance-based platform, the changes in the CI value can also be interpreted as morphological changes in the cells. The decrease in the CI value associated with the highest CHX concentrations used could result from both the diminished cell proliferation rate and cell morphological changes, namely the decrease in the number of cell divisions and the appearance of small round-shaped cells.

Many cytotoxic agents modulate the intricate balance between cell proliferation and cell death (33). Cell death occurs through a spectrum of morphological and biochemical pathways culminating in apoptosis, necrosis or autophagy. Reduced viability often results from diminished cell proliferation or cell cycle arrest. The suggested mechanisms underlying CHX-induced cytotoxicity are connected with the inhibition of collagen synthesis (12,26), the inhibition of protein synthesis (14,27) or the induction of reactive oxygen species (ROS) (34). Faria *et al* (24,25) found that only at the concentration of 0.00025% CHX there was no sign of apoptosis and necrosis. The increase in the number of apoptotic cells was concentration-dependent, but starting from 0.004% CHX there was no difference compared with the control group. The higher concentration of CHX induced cell necrosis. Chang *et al* (14) used 5% CHX and indicated that it was cytotoxic to periodontal ligament cells at the concentration of 0.0001% or greater, and inhibited protein synthesis at the 0.005% concentration. The protein synthesis was almost completely inhibited by the concentration of >0.05%, as was the mitochondrial activity of the human periodontal ligament cells, which was completely inhibited by 0.125% CHX. CHX may also induce cell death by apoptosis and necrosis via endoplasmic reticulum stress (25). Based on studies conducted on human osteoblastic and murine endothelial cells and fibroblasts, Gianelli *et al* (7) suggested that CHX exerts toxic through the induction of apoptotic and auto/necrotic cell death and involves the reduction of mitochondrial membrane potential, an increase in intracellular Ca^{2+} levels and oxidative stress.

Our results suggest that CHX induced cell cycle arrest at the S phase. Both the number of cells at the G_0/G_1 and G_2/M phase decreased, while the number of cells at the S phase increased.

Hidalgo *et al* (8) observed that CHX exerted an inhibitory concentration-dependent effect on DNA synthesis from the concentration as low as 0.0001% in dermal fibroblasts. In this study, the changes in the cell cycle were observed at the concentration of 0.04% (minimum). Looking at this discrepancy, one can speculate that CHX exhibits a different degree of cytotoxicity towards different cell types. However, this difference may also be due to the different times of cell exposure to CHX. Hidalgo *et al* (8) incubated cells with CHX for 3, 6, 8 and 24 h, whereas we incubated the cells with CHX for 15 min. These could also be reasons for differences in DNA synthesis observed in our study. Hidalgo *et al* (8) used 5-bromodeoxy-uridine (BrdU), a thymidine analogue that is incorporated into the cells during the DNA synthetic phase of replicating cells (during the S phase of the cell cycle). They observed a significant decrease in BRdU incorporation that occurred at the concentration of 0.0001% CHX, which reflects the decrease in the number of cells in the S phase. We observed the concentration-dependent accumulation of cells in the S phase together with a decrease of cells in the G_2/M phase following stimulation with CHX, which indicates that these cells do not seem to re-enter the cell cycle. It cannot be excluded that CHX is able to modify cell culture conditions so that quiescent S phase cells appear. Thus, the accumulation of inactive cells in the S phase would accompany the decreasing frequency of BrdU-positive cells. However, further research is warranted to confirm these findings.

Cell cycle arrest is often followed by resumed entry into the cell cycle or cell demise via apoptosis. Our results suggest that cells were arrested in the S phase to repair the CHX-induced DNA damage, and that some of the damage was not repaired causing the cells to undergo apoptosis.

In our study, we did not detect any apoptotic symptoms in the CHX-stimulated cells at the concentration below 0.01%. The percentage of apoptotic cells increased to 9.0% of cells at the highest concentration. The number of apoptotic cells was assessed based on the percentage of sub-G1 (<2N DNA) fraction in HGFs, the internucleosomal DNA fragmentation being one of the hallmarks of apoptosis. As DNA oligomers are extracted during cell staining, apoptotic cells can be identified on DNA content frequency histograms, as cells with fractional sub-G1 DNA content. However, the sub-G1 DNA content cannot be used as the sole marker of apoptotic cells, as DNA fragmentation to the oligo- or mono-nucleosomal-size fragments does not always take place during apoptosis (35).

To summarize, in conditions similar to those observed *in vivo*, the low CHX concentration has a different effect on gingival fibroblasts than the high concentration. However, even this low concentration has a greater influence on cells than the untreated controls. The low CHX concentration has minimal cytotoxicity, as it decreases proliferation without inducing morphological changes and apoptosis.

These findings suggest a different clinical protocol for patients with improper oral hygiene and patients after surgical treatment. The low CHX concentration can have antimicrobial activity and does not influence wound healing. It was found that 0.004% CHX in toothpaste inhibits bacterial colonization and growth on an enamel surface; however, even this low concentration of CHX was higher than the minimal concentration needed for the elimination of *Streptoccocus mutans* (36). The minimal inhibitory concentration (MIC) of CHX on periodontal

pathogens is 0.0012%. In addition, the penetration of CHX into the biofilm seems to be easier at lower concentrations. The compact matrix inhibits the diffusion of solutes, such as CHX into the biofilm. It is possible that conformational changes in biofilm structure, such as the opening up of the water channel, could assist in the diffusion of CHX into deeper layers. It was observed after using CHX at the concentration of 0.05% , but not at the concentration of 0.2% (37). Our previous clinical study also confirmed the effectiveness of a low CHX concentration (0.04%) in he subgingival irrigation in patients treated for chronic periodontal disease (38).

In conclusion, the aim of this study was to evaluate the effects of different concentrations of CHX on HGFs. The low concentration (0.002%) of CHX does not interfere with the proliferation and morphology of gingival fibroblasts. The higher concentration (≥0.04%) of CHX inhibits cell proliferation and, to a certain extent, affects cell morphology. Thus, the application of CHX in the post-surgical antiseptic treatment of the oral cavity should be limited.

Acknowledgements

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