Investigating the effect of antiseptic solution on the release of interleukin-6 and transforming growth factor beta 1 from human gingival fibroblasts using wound healing assays

Tanja Berner1), Ken Nakahara3), Eizaburo Kobayashi1), Akira Tanaka3), Yoichi Taniguchi4), Tateyuki Iizuka3), and Kosaku Sawada3)

1) Department of Oral and Maxillofacial Surgery, The Nippon Dental University School of Life Dentistry at Niigata, Niigata, Japan
2) Center of Dental Medicine, University of Zurich, Zurich, Switzerland
3) Advanced Research Center, The Nippon Dental University School of Life Dentistry at Niigata, Niigata, Japan
4) Department of Periodontology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan
5) Department of Cranio-Maxillofacial Surgery, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland

Abstract: This in vitro study evaluated the effect of different antiseptics and different concentrations thereof in a model of wound healing using human gingival fibroblasts. The fibroblasts were rinsed with four different antiseptic solutions: sodium hypochlorite (HYP), hydrogen peroxide (H2O2), chlorhexidine digluconate (CHX), and benzalkonium chloride (BC). The effect on the release of interleukin-6 (IL-6) and transforming growth factor beta 1 (TGF-β1) was investigated using enzyme-linked immunosorbent assays (ELISAs). In addition, the effects of the antiseptics on wound healing at 1, 12, 24, and 48 h were assessed through a wound healing assay. The viability of the fibroblasts rinsed with antiseptics was investigated with respect to the concentrations inhibiting cell growth by 50% (IC50), 25% (IC25), and ≤2% (IC1). A statistically significant increased release of IL-6 was obtained with BC IC25 after 12, 24, and 48 h (P < 0.01). For TGF-β1, no significant release was found for CHX IC25 after 24 and 48 h or for IC50 and IC25 after 12 h. There was no significant effect on wound healing capacity for CHX or for BC IC50 and IC25. This study demonstrated that antiseptic rinses of human gingival fibroblasts alter the release of IL-6 and TGF-β1 and impact wound healing capacity, with both BC and CHX conferring neutral effects.

Keywords: antiseptic, gingival fibroblast, IL-6, TGF-β, wound healing

Introduction

Wound healing is a complex biological process that consists of several overlapping phases and is influenced by many factors [1]. Delayed wound healing increases the risk of infections, which are often challenging to treat and may lead to severe medical and dental problems. The oral cavity harbors a wide range of bacterial species, and controlling their growth or elimination requires the use of several different types of antiseptics and disinfectants [2-4]. In the maxillofacial region, antiseptics contribute to the prevention of infection, such as the prophylactic use of antibiotics before oral surgery and the prevention of chemotherapy-induced oral mucositis [5,6]. Antiseptic solutions are also prescribed for the treatment of active infections, such as periodontitis and peri-implantitis, and for patients with bisphosphonate-related osteonecrosis of the jaw [7-9]. Sodium hypochlorite (HYP), hydrogen peroxide (H2O2), chlorhexidine digluconate (CHX), and benzalkonium chloride (BC) are commonly used for oral cavity rinses. The cytotoxic effects on, for example, bone cells, neuronal and Schwann cells, epithelial cells, and fibroblasts [13-18]. Following their migration into the wound site, both fibroblasts and immunoregulatory cells participate in wound healing [1,19]. In a previous study, the cytotoxicity of various antiseptic solutions, including the stimulation by low concentrations of CHX of TGF-β, and, in turn, the expression of their target genes in human fibroblasts was demonstrated [17]. These results suggested that the effects of antiseptic solutions go beyond those affecting cell viability to include the regulation of gene expression by the cytokines and the growth factors responsible for cell growth and inflammation. The most important functions of fibroblasts in wound healing include collagen production and the release of growth factors [20]. Transforming growth factor beta 1 (TGF-β1) is a key cytokine in the wound healing process since it influences the proliferation of fibroblasts and promotes collagen synthesis, inflammatory response, and angiogenesis [21,22]. Meanwhile, interleukin-6 (IL-6) plays an important role in the regulation of inflammation, stimulating the formation of new blood vessels, collagen production, and leukocyte infiltration [23,24]. Both TGF-β1 and IL-6 then play important roles in the process of wound healing.

In this study, the effect of four commonly used antiseptic solutions on human gingival fibroblasts is investigated. The effects of different concentrations of these solutions on the release of IL-6 and TGF-β1, as a representative cytokine and a representative growth factor, respectively, are investigated, as are the effects on wound healing.

Materials and Methods

Cell culture

Cells from the normal human gingival fibroblast cell line HGF-1 (ATCC CRL-2014; American Type Culture Collection, Manassas, VA, USA) were cultured in a complete growth medium containing phenol red-free Dulbecco’s modified Eagle’s medium (Fujifilm Wako Pure Chemical, Osaka, Japan), 10% fetal bovine serum (Sigma-Aldrich Chemie, Taufkirchen, Germany), and 1% antibiotic-antimycotic mixture, at 37 °C in a humidified atmosphere of 95% air and 5% CO2.

Antiseptic solutions

Four antiseptic solutions were selected for this study: HYP (10%; Neo Dental Chemical Products, Tokyo, Japan), BC (10%; Yoshida Pharmaceutical, Tokyo, Japan), H2O2 (2.5%; Kenei Pharmaceutical, Osaka, Japan), and CHX (0.2%; Glaxo Smith Kline Consumer Healthcare, Bern, Switzerland). Prior to their use, the antiseptics were filter-sterilized (0.22-mm pore diameter; Merck Millipore, Billerca, MA, USA) and diluted in sterilized water.

Determination of inhibitory and non-cytotoxic concentrations

To determine the effect of the antiseptics on cell viability, HGF-1 cells (1 × 105 cells/mL) were seeded in 96-well plates and then incubated at 37 °C in 5% CO2 for 24 h. After the medium was aspirated, the cells were exposed to the antiseptic solutions at the chosen concentrations for the indicated time. Subsequently, the antiseptic solutions were removed, and the cells...
were washed with phosphate-buffered saline (PBS) before 120 μL of cell culture medium containing 20 μL of MTS solution (CellTiter 96 One Solution Cell Assay; Promega, Madison, WI, USA) was added to each well of the 96-well plate. Cell viability was determined 4 h later by measuring the absorbance of the cells at 490 nm on a microplate reader (Infinite 200; Tecan Group Ltd., Männedorf, Switzerland). The experiments were performed in triplicate, with three independent experiments for each condition. The data were normalized to the values of the antiseptic-free control (CON) samples.

The concentration of antiseptic inhibiting growth by 50% (IC50) was calculated using the following equation: IC50 = 10\[10^\log(A/B) \times (50 - C) / (D - C) + \log(B)]\], where A is the higher and B is the lower of the two concentrations that sandwich the IC50, C is the cell viability (%) at B, and D is that at A. The same method was used to calculate the 25% cell growth inhibitory concentration (IC25). The concentration resulting in ≤25% growth inhibition (IC25) was similarly determined and defined as the non-cytotoxic concentration.

The impact of all three concentrations was investigated using enzyme-linked immunosorbent assays (ELISAs), which allowed for protein quantification, and a wound healing assay.

**ELISA protein quantification**

To investigate the effect of the antiseptic solutions on the protein secretion by gingival fibroblasts, the cells were exposed to the four different antiseptic solutions (HYP, BC, H2O2, and CHX) at three different concentrations (IC25, IC50, IC75) for 1, 12, 24, and 48 h. At each time point, the culture supernatant was sampled for use in IL-6 and TGF-β1 ELISAs (R&D Systems, Minneapolis, MN, USA), performed according to the manufacturer’s protocol. Briefly, 100 μL of assay mix and 50 μL of the sample were incubated at room temperature in 96-well plates pre-coated with an antibody. Two hours later, the wells were washed four times with washing buffer, incubated for 2 h with peroxidase-conjugated antibody solution, and then washed again, before 200 μL of substrate solution was added for 30 min followed by 50 μL of stopping solution for another 30 min. The absorbance was measured at 450 nm on a microplate reader. All samples were measured in triplicate and three independent experiments were performed.

**Wound healing assay**

The proliferation and the migration of human gingival fibroblasts were monitored in a wound healing assay (CytoSelect 24-Well Wound Healing Assay; Cell Biolabs, Inc., San Diego, CA, USA), and the results of a defined wound area were compared with those from a control scratch wound assay. To obtain an HGF-1 cell monolayer with a defined wound area, a cell solution (0.5 × 10^6 cells/mL) was poured alongside a plastic insert. Following overnight culture, the plastic insert was removed, leaving a defined cell-free area with a width of 0.9 mm. The wounded fibroblast cultures were exposed to the four different antiseptic solutions (HYP, BC, H2O2, and CHX) at their respective IC25, IC50, and IC75 values.

To track the cell proliferation and migration visually at the wound site, the cultures were photographed using the EVOS XL cell imaging system (Thermo Fisher Scientific, Waltham, MA, USA). Specifically, images were obtained 1, 12, 24, and 48 h after the exposure of the cells to the different antiseptic solutions at specified concentrations. The size of the wound gap was measured at five randomly chosen sites within the gap using ImageJ (National Institutes of Health, Bethesda, MD, USA). All samples were measured in triplicate and three independent experiments were performed.

**Statistical analysis**

Statistical analysis was carried out using SPSS (IBM, Tokyo, Japan). All measured data were analyzed using one-way analysis of variance (ANOVA) and the Bonferroni test (P < 0.01). All groups were homoscedastic (Levene, P > 0.05). As a result of the ANOVA, significant differences were detected between the groups. Each F-value is shown in the corresponding graph and while all combinations were tested using the Bonferroni test, the comparisons with the control samples are described.

**Results**

First, a quantitative analysis of the effect of different antiseptic solutions on the cell viability of human gingival fibroblasts was performed (Fig. 1). The effect of the different antiseptic solutions (HYP, BC, H2O2, and CHX) on the viability of human gingival fibroblasts showed a decrease in cell viability. The concentrations producing decreases are shown in Table 1 and were used in the subsequent experiments.

**ELISA protein quantification**

The release of IL-6 and TGF-β1 in response to the antiseptic solutions, at the concentrations determined in the previous experiment (Table 1), was investigated. One hour of exposure to any of the antiseptic solutions did not result in significant differences compared with the antiseptic-free control samples (CON; Fig. 2A). However, in cultures exposed for 12, 24, and 48 h to HYP and H2O2, IL-6 release was significantly reduced (P < 0.01) compared with that in the CON (Fig. 2B-D). Exposure to BC IC25 and IC50 for 12, 24, and 48 h resulted in a significant increase in IL-6, while a significant reduction occurred in cells incubated with BC at its IC50 (P < 0.01; Fig. 2B-D). In the CHX IC25 sample, IL-6 release was the same as that obtained in the CON sample at 12 h but was significantly lower at 24 and 48 h (P < 0.01) (Fig. 2B-D). In contrast, IL-6 release in response to CHX IC50 was the same as that of the CON at all time points (Fig. 2).

A significant reduction of TGF-β1 release (P < 0.01) was determined...
in the H2O2, P < 0.01; Fig. 3B-D). In cells exposed to CHX IC50, the CON (24, and 48 h, TGF-β1 release was reduced in the BC group compared with the CON (P < 0.01; Fig. 3A, B), while at 24 and 48 h, it was the same (Fig. 3C, D). In the CHX IC25 and CHX IC100 cultures, TGF-β1 release was significantly increased after 1 h but was significantly decreased after 24 and 48 h (P < 0.01; Fig. 3A, C, D).

Fig. 3 ELISA quantification of the release of transforming growth factor-β1 (TGF-β1) from human gingival fibroblasts rinsed with HYP, BC, H2O2, and CHX after (A) 1, (B) 12, (C) 24, and (D) 48 h. F (12, 26) = (A) 2626.874, (B) 8115.296, (C) 3920.792, (D) 7478.330, P < 0.01. **significantly different compared with the control sample (both P < 0.001).

Table 1 Concentrations of the antiseptic solutions and the effects on cell growth: 2% (IC2), 25% (IC100), and 50% (IC50) inhibition

| Antiseptic: | HYP (sodium hypochlorite) | BC (benzalkonium chloride) | H2O2 (hydrogen peroxide) | CHX (chlorhexidine digluconate) |
|------------|---------------------------|-----------------------------|--------------------------|-------------------------------|
| IC2 (Cell viability = 98%) | 0.01563%                   | 0.00078%                    | 0.00781%                 | 0.00039%                     |
| IC25 (Cell viability = 75%) | 0.01822%                   | 0.00089%                    | 0.00891%                 | 0.00156%                     |
| IC50 (Cell viability = 50%) | 0.02094%                   | 0.00102%                    | 0.02552%                 | 0.00241%                     |

Wound healing assay

The four antiseptic solutions were also tested for their effects on the wound healing capacity of gingival fibroblasts, using the concentrations shown in Table 1.

As shown in Fig. 4, after 48 h, wound closure was achieved in the CON sample as well as in the CHX IC50 and, to a lesser extent, the BC IC25 cultures. In the wounded cultures exposed to HYP IC50, a loss of attachment was observed such that only a few migrating cells were seen in the wound area. In the H2O2 (IC50) cultures, the wound closure was only partial.

The wound healing capacity was measured as the distance between the cells and is shown in Fig. 5. In the gingival fibroblasts exposed to HYP IC50, the wound healing capacity was significantly reduced at all time points compared with the respective controls (P < 0.01) (Fig. 5A). The wound healing capacity of the HYP IC25, and IC100 cultures after 24 and 48 h was significantly lower than that of the CON culture (P < 0.01; Fig. 5A). In the BC IC25 culture, the wound healing capacity was also significantly reduced after 24 and 48 h (P < 0.01; Fig. 5B), while this was not the case in the BC IC100 cultures. A significant difference compared with the CON was determined in the H2O2-exposed cultures after 24 and 48 h, regardless of the antiseptic concentration (P < 0.01; Fig. 5C). Interestingly, none of the CHX concentrations significantly affected the wound healing capacity of the HGF-1 cells compared with the CON at any time point (Fig. 5D).

Discussion

The current study demonstrates that the release of IL-6 and TGF-β1 by human gingival fibroblasts, and the wound healing capacity of these cells, differs depending on the antiseptic solution and the exposure concentration. While the inhibitory and non-cytotoxic concentrations were determined after 24 h, the cell cultivation was continued and the results after 48 h were also included in this study, since cell activity was still observed after 24 h. Lin et al. [23] reported that IL-6 plays an important role in wound healing in terms of stimulating collagen synthesis and angiogenesis, while it is also a key cytokine regulating acute immune response [25]. However, Dongari-Bagtzoglou et al. [26] found that IL-6 levels are increased in...
patients with periodontal disease. The results of this study show that anti-
septic solutions impact the release of IL-6 by human gingival fibroblasts, with BC IC1 and IC2, having a quantitatively greater effect after 12, 24, and 48 h of exposure than the corresponding control samples. In contrast, the levels of IL-6 released by the CHX-exposed cultures were similar to those by the control cultures, while the IL-6 release induced by HYP and H2O2 at any concentration was significantly lower than that measured in the CON. On this basis, it can be assumed that HYP and H2O2 might result in an inhibition of the release of IL-6 after 12, 24, and 48 h, while further investigation is needed to elucidate these findings.

Wound healing is a complex process involving the interaction of various factors, including TGF-β1, which functions as a regulatory protein with profibrotic activity [27]. TGF-β1 stimulates extracellular matrix production, especially collagen types I and III, but also inhibits inflammatory responses [28,29]. However, the overexpression of TGF-β1 may lead to pathologic scar formation [30,31]. In this study, an increase in the release of TGF-β1 was identified for CHX IC2 after 12 h compared with that of the CON, while the release was equal to that of the CON after 24 and 48 h.

The oral cavity contains a wide range of microflora, which complicates wound healing [32]. Antiseptic solutions are widely prescribed to prevent or combat infections in the oral and maxillofacial regions. However, adverse effects have also been described, with Sawada et al. [33], for example, reporting that antiseptics reduce the viability of bone cells. In the present study, the effects of four antiseptic solutions on human gingival fibroblasts in a wound healing assay were examined. In endodontic treatments, HYP is commonly used due to its antimicrobial action and its ability to eliminate necrotic tissue [34]. However, the fibroblasts exposed to HYP in the wound healing assay were detached, and effective wound closure was prevented, as shown in Fig. 5.

BC has long been used as a disinfectant, not only in oral cavity solutions, such as mouthwashes, but also in hand and face soaps [35]. In this study, BC IC1; and IC2 induced a significant increase in IL-6 (Fig. 2), but the effects on wound healing were not significantly different to those in the control sample (Fig. 5B). Specifically, although continuous cell growth was detected in gingival fibroblasts rinsed with BC, the wound area was not as dense as in the CON or CHX cultures, as shown in Fig. 5.

De Saint Jean et al. [36] showed that BC inhibits cell growth and induces apoptosis in conjunctival cells. H2O2 is mostly used in endodontic procedures such as root canal treatment as a disinfectant. However, it is effective for wound debridement, its antibacterial effect is not particularly strong [37,38]. While the human gingival fibroblast monolayers exposed to H2O2, IC1, and IC2 showed some shrinkage of the wound area, the wound healing was clearly inadequate (Fig. 5C). Meanwhile, in the cultures treated with H2O2, IC2, there was no change in the width of the wound area over time. In their study of CHX, Tsourounakis et al. [39] showed that a concentration of 0.012% had some antibacterial capacity but led to a reduction in cell migration and had negative effects on cell viability. In contrast, no difference was found in the wound healing capacity of human gingival fibroblasts rinsed with CHX at concentrations between 0.00039% and 0.00241%.

A comparison of the IL-6 concentration with fibroblast migration showed a tendency toward a low release of IL-6 and unfavorable wound healing for HYP and H2O2. For CHX IC1, there was minimal difference to the control sample (Fig. 5D). CHX induced levels of IL-6 and TGF-β1 release that were similar to that of the control sample at 12, 24, and 48 h, with an additional transient increase of TGF-β1 by CHX IC2 at 12 h. In their review, Patel et al. [40] noted the frequent prescription of CHX in patients with bisphosphonate-related osteonecrosis of the jaw. In fact, the American Association of Oral and Maxillofacial Surgeons (AAOMS) recommends the use of CHX as an oral mouthwash for patients with medication-related osteonecrosis of the jaw [41]. Within the limitations of this in vitro study, the results support these recommendations, as the fibroblasts rinsed with CHX released higher amounts of TGF-β1, which would stimulate the formation of replacement tissue as a protective layer, including, it can be assumed, in the oral cavity.

Among the limitations of this study were the small sample size and the difficulty in disentangling the wound-healing-promoting activities of IL-6 and TGF-β1. Furthermore, the effects of the tested antiseptics were not examined on the bacteria found in the oral cavity. The concentrations of the four different antiseptic solutions were analyzed, as shown in Fig. 1, and the precise concentrations that were used for further studies are shown in Table 1 on this page. It is clear that low concentrations have a high impact on the cell viability, which might have a negative effect on wound healing. Thus, further investigations are needed to determine the optimal concentration of the antiseptic solution, that is, one that demonstrates antibacterial activity, including minimum inhibitory (MIC) and minimum bactericidal concentration (MBC), but without negative effects on wound healing.

In addition, further studies using animal models are needed to verify the results, while it is also necessary to investigate other proteins, such as TNF-α, VEGF, or type I collagen, including in terms of the detection of apoptotic cells, to examine the accurate effect of antiseptic solutions on the complex regulation process of wound healing.

In conclusion, despite the limitations of the present study, the results demonstrate the potentially favorable effects of BC and CHX on wound healing and the lack of adverse effects on cells exposed to HYP and H2O2.
297

27. Leask A, Abraham DJ (2004) TGF-beta signaling and the fibrotic response. Faseb J 18, 816-827.
28. Silvestro-Ruiz KG, Martinez AE, Garlet GP, Barbosa CF, Silva JS, Cicarelli RMB et al. (2007) Opposite effects of bFGF and TGF-beta on collagen metabolism by human periodontal ligament fibroblasts. Cytokine 39, 130-137.
29. Safari SM, Kazemi B, Esmaeili M, Fallah B, Modarresi A, Mir M (2008) Effects of low-level He-Ne laser irradiation on the gene expression of IL-1α, TNF-α, IFN-γ, TGF-β, bFGF, and PDGF in rat’s gingiva. Lasers Med Sci 23, 331-335.
30. Shah M, Foreman DM, Ferguson MW (1995) Neutralisation of TGF-β1 and TGF-β2 by exogenous addition of TGF-β1 to cutaneous rat wounds reduces scarring. J Cell Sci 108, 985-1002.
31. Colwell AS, Phan TT, Kong W, Longaker MT, Lorenz PH (2005) Hypertrophic scar fibroblasts have increased connective tissue growth factor expression after transforming growth factor-β stimulation. Plast Reconstr Surg 116, 1387-1390.
32. Caglar E, Kargul B, Tanboga I (2005) Bacteriotherapy and probiotics’ role on oral health. Oral Dis 11, 131-137.
33. Sawada K, Fujisaka-Kobayashi M, Kobayashi E, Schaller B, Miron RJ (2016) Effects of antiseptic solutions commonly used in dentistry on bone viability, bone morphology, and release of growth factors. J Oral Maxillofac Surg 74, 247-254.
34. Heling I, Rotstein I, Dinur T, Szwee-Levine V, Steinberg D (2001) Bactericidal and cytotoxic effects of sodium hypochlorite and sodium dichloroisocyanurate solutions in vitro. J Endod 27, 278-280.
35. Percival SL, Finnegan S, Donelli G, Vuotto C, Rimmer S, Lipsky BA (2016) Antiseptics for treating infected wounds: efficacy on biofilms and effect of pH. Crit Rev Microbiol 42, 293-309.
36. De Saint Jean M, Brignole F, Bringuier AF, Bauchet A, Feldmann G, Baudouin C (1999) Effects of benzalkonium chloride on growth and survival of chang conjunctival cells. Invest Ophthalmol Vis Sci 40, 619-630.
37. Nishikori R, Nomura Y, Sawajiri M, Masuki K, Hirata I, Okazaki M (2008) Influence of chlorhexidine diacetate and cell cycle of human gingival fibroblasts. J Dent 36, 993-998.
38. Mirfani H, Abbasaedegan A, Ranjbar MA, Azar MR, Geramizadeh B, Torahi S et al. (2015) Antibacterial and toxic effect of hydrogen peroxide combined with different concentrations of chlorhexidine in comparison with sodium hypochlorite. Dent Shiraz Univ Med Sci 16, 349-355.
39. Tsounakis I, Palaiologou-Gallis AA, Stoute D, Maney P, Lallier TE (2013) Effect of chlorhexidine mouthwashes on gingival fibroblast survival and migration. J Periodontol 84, 1211-1220.
40. Patel V, McLeod NMM, Rogers SN, Brennan PA (2011) Bisphosphonate osteonecrosis of the jaw—a literature review of UK policies versus international policies on bisphosphonates, risk factors and prevention. Br J Oral Maxillofac Surg 49, 251-257.
41. Ruggiero SL, Dodson TB, Fantasia J, Goodday R, Aghalase T, Mehrotra B et al. (2014) American association of oral and maxillofacial surgeons position paper on medication-related osteonecrosis of the jaw 2014 update. J Oral Maxillofac Surg 72, 1938-1956.