RESEARCH ARTICLE

Cytotoxic effects of octenidine mouth rinse on human fibroblasts and epithelial cells – an in vitro study

J. Schmidt¹, V. Zyba², K. Jung³, S. Rinke⁴, R. Haak¹, R. F. Mausberg², and D. Ziebolz¹

¹Department of Cariology, Endodontontology and Periodontology, University of Leipzig, Leipzig, Germany, ²Department of Preventive Dentistry, Periodontology and Cariology, University Medical Centre Goettingen, Goettingen, Germany, ³Department of Medical Statistics, University Medical Centre Goettingen, Goettingen, Germany, and ⁴Department of Prosthodontics, University Medical Centre Goettingen, Goettingen, Germany

Abstract

Objectives: This study compared the cytotoxicity of a new octenidine mouth rinse (MR) against gingival fibroblasts and epithelial cells with different established MRs. Methods: The following MRs were used: Octenidol (OCT), Chlorhexidine 0.2% (CHX), Listerine (LIS), Meridol (MER), Betaisodona (BET); and control (medium only). Human primary gingiva fibroblasts and human primary nasal epithelial cells were cultivated in cell-specific media (2 x 10⁵ cells/ml) and treated with MR for 1, 5, and 15 min. Each test was performed 12 times. Metabolism activity was measured using a cytotoxicity assay. A cellometer analyzed cell viability, cell number, and cell diameter. The data were analyzed by two-way analysis of variance with subsequent Dunnett’s test and additional t-tests. Results: The cytotoxic effects of all MRs on fibroblasts and epithelial cells compared to the control depended on the contact time (p < 0.001). OCT and BET showed less influence on cell metabolism in fibroblasts than other MRs. OCT also demonstrated comparable but not significant results in epithelial cells (p > 0.005). Cell numbers of both cell types at all contact times revealed that OCT showed a less negative effect (p > 0.005), especially for epithelial cells compared to CHX after 15 min (p < 0.005). OCT and BET showed the best results for viability in fibroblasts (p > 0.005), but MER showed less influence than OCT in epithelial cells (p < 0.005). Conclusions: OCT is a potential alternative to CHX regarding cytotoxicity because of its lower cell-toxic effect against fibroblasts and epithelial cells.

Keywords

Mouth rinse, cytotoxicity, oral cells

Introduction

Antimicrobial mouth rinses (MRs) help support oral biofilm management. MRs contain antimicrobial agents, the so-called antiseptics, which are expected to work within the oral cavity in a targeted and highly effective manner without causing side effects (McDonnell & Russell, 1999; Müller & Kramer, 2008; Schulman, 1993). Antiseptics are anti-infective substances that destroy (i.e. bactericidal effect) or inhibit (i.e. bacteriostatic effect) microorganism growth after topical application (McDonnell & Russell, 1999). Generally, MRs support mechanical plaque control within the oral biofilm (Fine et al., 2007). MRs are applied to prevent or slow biofilm formation and the multiplication of pathogenic bacteria.

In addition to the bacteriostatic, bactericidal, fungistatic, and/or fungicidal activities of MRs, a specific effect against pathogenic bacteria is intended but not necessarily achieved (Slots, 2002). MR use clearly reduces germs, but the substantivity of the MR is also important (Cummins & Creeth, 1992). Substantivity is meant to be the residence time and activity of a substance in an antimicrobiotically effective concentration. However, MRs must be toxicologically harmless even during long-term therapy and not prone to trigger allergies or absorption via oral mucous membranes or the gastrointestinal tract. The cytotoxic effects of the MR must also be considered. This aspect is vitally important to avoid the inhibition of healing processes and minimize toxic effects to host tissue.

Chlorhexidine (CHX) 0.2% is the gold standard MR (Jenkins et al., 1994; Löe & Schiott, 1970). CHX has a high degree of substantivity on the negatively charged surfaces in the oral cavity (Jenkins et al., 1994; Tomás et al., 2008). CHX has cytotoxic effects on various body cells, including epithelial cells (Millhouse et al., 2014), fibroblasts (Eick et al., 2011), and stem cells (Park et al., 2014).

Known MR alternatives also have cytotoxic effects, but these alternatives are less effective against bacteria. In dentistry several MRs are established using different active ingredients. One group of active MR ingredients is composed of essential oils, e.g. Listerine (LIS). The substantivity of this group is considerably lower than CHX (Otten et al., 2010). Alcohol is most commonly used as a solubilizer between the
essential oils and water (21.6–26.9 Vol%) in this group. Alcohol is likely responsible for the cytotoxic effects on gingival fibroblasts (Eick et al., 2011) and stem cells (Park et al., 2014) that were described for LIS. Another group of active MR ingredients are based on amine and stannous fluorides, e.g. Meridol (MER). The organic amine fluoride stabilizes the antimicrobial stannous fluoride (Arweiler et al., 2001; Neuschil et al., 1995). Eick et al. demonstrated pronounced cytotoxic effects of MER on gingival fibroblasts, which was similar to CHX and LIS (Eick et al., 2011). Another example of oral antiseptics is povidone iodine (PVP-iodine), a water-soluble complex of iodine and polyvinylpyrrolidone (trade name Betasodona: BET). Povidone iodine is a compound from the group of iodophores. Cytotoxic effects on epithelial cells were confirmed for BET despite a low concentration of active ingredients, which was significantly lower than the clinically applied concentration (Sato et al., 2014).

Studies to develop a MR with an efficacy that is comparable to CHX but with better oral biocompatibility are ongoing. This research led to the identification of octenidine dihydrochloride, trade name Octenodil (OCT). The microbiostatic and microbiocidal effectiveness of OCT is 10 times higher than CHX (Sedlock & Bailey, 1985), and this product also shows better biocompatibility, which characterizes the cellular and bacterial toxicities of topical antimicrobials (Müller & Kramer, 2008). Neither adverse effects nor allergic reactions are described in the literature. The high effectiveness of OCT supports its potential as an alternative to CHX. However, the available data for OCT are insufficient, with a particular lack of studies on cytotoxicity.

Therefore, our investigations compared the toxic effect of the new MR OCT on fibroblasts and epithelial cells to CHX and the other MRs, such as LIS, MER, and BET, which are well established in the field of dental medicine. Cytotoxicity, cell number, viability, and mean cell diameter were determined in an in vitro investigation after treatment of the cells with each of the tested MRs.

We hypothesized that OCT would exhibit a lower cytotoxic potential than CHX.

Materials and methods

The present investigation was an experimental, controlled, six-arm in vitro study of primary human cell to investigate the cytotoxicity of several antiseptic MRs.

Materials

Cells

Cryoconserved primary human gingival fibroblasts (HGFIBs; order number 1210412, Proviro GmbH, Berlin, Germany) and primary human nasal epithelial cells (HNEPCs; order number 1210711, Proviro GmbH, Berlin, Germany) were the basic materials for cell cultivation. These primary human cells were cultivated as described below in order to obtain the cells to be used in the experiments.

Freezing was performed in aliquots (1 × 10^6 cells, respectively) under standardized conditions in Cryo-SFM-Medium (order number 2040102, Proviro GmbH, Berlin, Germany) in liquid nitrogen. The aliquots were thawed in a water bath at 37 °C for the test series.

Immunohistological analyses revealed that HGFIBs were positive for the HGFIB-specific receptor CD90/Thy-1, and HNEPCs were positive for keratin. Cells were subjected to infection serology tests for bacteria, fungi, mycoplasms, HIV-DNA, and hepatitis B/C-DNA, and all cells were classified as negative.

Cell culture medium

The following media were used for cell culture: fibroblast growth medium (order number 2010401, Proviro GmbH, Berlin, Germany) that contained 10% fetal calf serum (FCS), 50 ng/ml amphotericin B, and 50 μg/ml gentamicin; and epithelial cell growth medium (Airway epithelium cell growth medium, order number 2030701, Proviro GmbH, Berlin, Germany) that contained 0.4% bovine pituitary extract (BPE), 5 μg/ml epidermal growth factor (EGF), 0.5 μg/ml hydrocortisone, 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 0.1 ng/ml retinoic acid, 6.7 ng/ml T3, and 5 μg/ml insulin.

These media were used during initial cell cultivation.

Cell cultivation

Cell cultivation was performed using standardized methods under sterile conditions based on the above-mentioned primary cells. Cell suspensions were added to 10 ml fibroblast growth medium or epithelial cell growth medium and centrifuged for 5 min at 250 × g at room temperature. The cells were resuspended and transferred to cell culture bottles. HGFIBs were seeded at a concentration of 4000 cells/cm^2, and HNEPCs were seeded at a concentration of 6000 cells/cm^2. The cells were cultivated in a constant nitrogen environment of 5% at 37 °C and saturated humidity in a CO₂ incubator. Culture medium was replaced by fresh culture medium every 2–3 d. Adherent growing cells were passaged after a 70% to 80% confluency of the culture flask surface was obtained. The passage of HGFIBs was performed using a sterile trypsin-EDTA solution (Passage Kit 2, order number 2040002, Proviro GmbH, Berlin, Germany), and HNEPCs were treated with dispase II (Passage Kit 3, order number 2040003, Proviro GmbH, Berlin, Germany) and incubated for 15 min under the same conditions. The removed cells were transferred to a 15-ml centrifuge tube and centrifuged for 5 min at 250 × g at room temperature. The supernatant was discarded, and the remaining cell pellet was resuspended in 5 ml of culture medium for cell number quantification. HGFIBs from passage 6 and 7 and HNEPCs from passage 4 were used for experiments.

Mouth rinses

Chlorhexidine (CHX; chlorhexidine digluconate, ready-to-use solution 0.2%, Engelhard Arzneimittel GmbH & Co. KG, Niederdorfelden, Germany), amine/stannous fluoride (MER: Meridol, GABA GmbH, Lörrach, Germany), a combination of essential oils, methyl salicylate, sodium citrate, and benzoic acid (LIS: Listerine® Coolmint, Pfizer Consumer Healthcare GmbH, Karlsruhe, Germany), and povidone iodine
(BET: Betaisodona, Mundipharma GmbH, Limburg/Lahn, Germany) were used as the antibacterial active ingredients in the respective commercial MRs for comparisons with octenidine (OCT: Octenidol, Schülke GmbH, Norderstedt, Germany) (Table 1). Oral antiseptics CHX, MER, LIS, and OCT were sterile-filtered and used without dilution in subsequent cell cultures. The BET suspension was diluted after sterile filtration in a 1:5 proportion with bidistilled water according to the manufacturer’s specifications. The preparations used in this study were commercially available MRs. Only LIS contained ethanol (21.6%). BET, MER, and OCT did not contain any ethanol. The control groups (CTRL) were treated with phosphate-buffered saline (PBS) without the addition of antiseptics.

Treated cells were resuspended after the determination of the cell number in fresh culture medium and transferred to 12-well plates according to the respective group and contact time. The cell concentration was adjusted to $2 \times 10^5$ cells/ml culture medium per well. The medium was exchanged after every 24 h, and light microscopy observed crystal formation. The wells were treated with 1 ml of the respective oral antiseptic after 96 h (Table 1). All tests were performed in duplicate and repeated 12 times. Each solution remained on the cells for 1, 5, or 15 min. Active ingredient solutions were aspirated. The wells were rinsed with 1 ml PBS for 30 s and refilled with 1 ml of fresh culture medium. Cells in the same culture conditions were cultivated for another 24 h. Finally, the prepared plates were analyzed using an MTT test and a computer-assisted cell analysis (Cellometer™ Auto T4).

**Cytotoxicity assay (MTT)**

The MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] test (proliferation assay) was used to analyze the metabolic activity of HGFIBs and HNEPCs. The MTT was described in detail by Mosmann (1983). The MTT solution (250 μl per well) was added to the medium. The 12-well plates were incubated for another 2 h under the same conditions. Enzymes of living cells reduced the yellow-colored tetrazolium salt to an insoluble blue-colored formazan product. Crystal formation was controlled visually with the help of a light microscope. Media with the MTT solution were aspirated carefully, and 1 ml of dimethylsulfoxide (DMSO) was added to every well to dissolve the crystals. The plates were incubated for another 15 min in the incubator. The optical density within the wells was measured using a spectrophotometer and an automated reader. A test wavelength of 555 nm and a reference wavelength of 690 nm were used for the HGFIBs. A test wavelength of 550 nm and a reference wavelength of 700 nm were used for the HNEPCs. Measurement results at a reference wavelength were subtracted from the test wavelength results (mean value calculation) for analysis purposes. The results of the control wells of every plate were also averaged and set as 100% because these cells were treated with pure PBS instead of the MRs to show their optimal growth. The results of the wells treated with oral antiseptics are shown as a percentage of the control wells.

**Cellometer analysis**

Determinations of the cell number, viability, and mean cell diameter were performed optically using the CellometerTM Auto T4 (Nexcelom Bioscience LLC, Lawrence, MA, USA). A software-supported application allowed the simultaneous determination of the number of living cells in relation to the total cell number (viability) stained with trypan blue. All cells from two associated wells were resuspended in 1 ml of the medium after centrifugation for cell counting purposes. This cell suspension (20 μl) was pipetted into a counting chamber, which was placed in the cellometer for automatic analysis. The dilution factor was set to 1, the cell type to HGFIB or HNEPC, and the cell diameter to 10–50 μm. Cell concentration was generated automatically, and the dilution factor was taken into account. The generated data (cell number, viability, and mean diameter) were transferred using Microsoft Excel.

**Statistical analysis**

The influence of the different MR solutions, contact time, and the interaction effect of these two factors on the measurements were investigated separately according to cell types using two-way analysis of variance plus interaction term ($p$ values are shown in Supplementary Table 1). In the case of a significant effect of the solution factor, each MR solution was compared to the control using Dunnett tests. Additionally, all MR solutions were compared to each other using paired $t$-tests. The $p$ values for the comparisons between the different MR for each of the examined parameters are shown in the Supplementary Tables 2–5.

The significance level was set to $\alpha = 5\%$ for each test. A significant difference for the paired comparisons of the MR solutions was only assumed when the $p$ value was below the Bonferroni-adjusted significance level of $0.05/10 = 0.005$. All testing procedures were conducted using the software SAS (version 9.1, SAS Institute), all figures were built using the software R (version 2.8, www.r-project.org).

| Group | Mouth rinses              | Active ingredient                             | Manufacturer                                                                 |
|-------|---------------------------|-----------------------------------------------|------------------------------------------------------------------------------|
| CHX   | Chlorhexidine digluconate | Chlorhexidinbis (digluconate)                 | Engelhard Arzneimittel GmbH & Co.KG, Niederdorfelden, Germany                 |
| MER   | Meridol                   | Amine/stannous fluoride (AmF/SnF₂)            | GABA GmbH, Lorrach, Germany                                                 |
| LIS   | Listerine                 | Cool mint: methyl salicylate, sodium citrate, and benzoic acid | Pfizer Consumer Healthcare GmbH, Karlruhe, Germany                           |
| BET   | Betaisodona               | Povidone-iodine (PVP-iodine)                  | Mundipharma GmbH, Limburg (Lahn), Germany                                    |
| OCT   | Octenidol                 | Octenidine dihydrochloride                    | Schülke GmbH, Norderstedt, Germany                                           |

Table 1. Overview of determined MR including anti-infective ingredients and manufacturer.
Results

Cell metabolism

The metabolic activity was significantly reduced after the treatment with the five MR (CHX, MER, LIS, BET, and OCT) in all test series (1, 5, and 15 min) (each $p < 0.0001$; Figures 1A and 2A) compared to the control cells (overall mean ± SE for control HGFIB: 0.36 ± 0.01; HNEPC: 0.19 ± 0.01). Comparisons of MRs with each other revealed no clear differences between the cytotoxic effect of individual solutions on the HGFIBs ($p > 0.005$) and HNEPCs ($p < 0.005$; Figures 1A and 2A). Analysis of variance revealed a significant influence of the MR and contact time, and a significant interaction of the MR and contact time on HGFIBs and HNEPCs (each $p < 0.001$). These results suggest that the effects of the MR used varied considerably depending on the contact time.

HGFIBs: The MR (CHX, MER, OCT, LIS, BET) showed a perceptible reduction in the metabolic activity of the HGFIBs depending on the contact time ($p < 0.0001$). There were no significant differences between groups in relation to the contact time ($p > 0.005$) (Table 2). The test series for OCT showed the lowest cytotoxic effect after 1 min. Comparable results were obtained for BET. The lowest cytotoxicity of the MR on HGFIBs was ranked as follows: OCT < BET < CHX = MER < LIS. The rankings for the 5-min contact time corresponded to the contact time of 1 min, except for BET and OCT: BET < OCT < CHX = MER = LIS. There was a change in the ranking according to the lowest cytotoxicity after a contact time of 15 min: BET < OCT < LIS < MER < CHX. The highest cytotoxicity for CHX was observed at the longest contact time. Overall, the metabolic activity was the highest level after OCT and BET treatment (Table 2).

Figure 1. Cell metabolism (A), cell number (B), cell viability (C), and cell diameter (D) of HGFIBs after incubation with several mouth rinses for different times (1, 5, 15 min).
HNEPC: A reduction in metabolic activity depended on the contact time of the MR solution \((p<0.0001)\). Overall, only small differences were observed between groups in relation to the contact time (Table 2). Except for the differences between CHX versus MER and CHX versus LIS \((p=0.0000)\) for the 15 min \((p=0.0000\) and \(0.0008\), respectively) and 5-min contact times \((p=0.0000)\) and CHX versus OCT for the 5-min contact time \((p=0.0000)\) no statistical significances were found \((p>0.005)\). OCT exhibited the highest cytotoxicity in the HNEPCs within the test series with

|           | HGFIBs | HNEPCs |
|-----------|--------|--------|
| MR 1 min  | 0.445 ± 0.014 | 0.217 ± 0.007 |
| MR 5 min  | 0.308 ± 0.014 | 0.215 ± 0.002 |
| MR 15 min | 0.342 ± 0.027 | 0.154 ± 0.008 |
| Control   | 0.308 ± 0.014 | 0.215 ± 0.002 |
| CHX 0.2%  | 0.018 ± 0.002 | 0.017 ± 0.002 |
| Meridol   | 0.018 ± 0.002 | 0.017 ± 0.002 |
| Listerine | 0.018 ± 0.002 | 0.017 ± 0.002 |
| Betaisodona | 0.021 ± 0.004 | 0.020 ± 0.005 |
| Octenidol | 0.022 ± 0.002 | 0.017 ± 0.001 |
mean ± SE for control HGFIB: 6.53 ± 0.18 [10^5 cells/ml]; MR treatment (CHX, MER, LIS, BET, and OCT) in all test series. CHX ranking was as follows: CHX ¼ 5 (after 1 min), CHX ¼ 4 (after 15 min) and at the longest contact time: OCT ¼ 4 (at the 5-min contact time) and at the longest contact time: OCT ¼ 4. More cells (HGFIB and HNEPC) were present in the OCT group after a contact time of 15 min (Table 2).

**Cell number**

The cell number was significantly reduced (p < 0.0001) after MR treatment (CHX, MER, LIS, BET, and OCT) in all test series (1, 5, and 15 min) compared to the control cells (overall mean ± SE for control HGFIB: 6.53 ± 0.18 [10^5 cells/ml]; HNEPC: 5.43 ± 0.11 [10^5 cells/ml]) (Figures 1B and 2B). The cell counts of HGFIBs and HNEPCs were significantly reduced without any dependence on the contact time when CHX, MER, and LIS were used. The negative effect of antiseptic therapy with OCT and BET was less pronounced. More cells (HGFIB and HNEPC) were present in the OCT series. Variance analyses showed a significant influence of the MR used and the contact time. However, a significant interaction between the type of the solution and the contact time on cell number was not observed for HGFIBs (p = 0.7337) or HNEPCs (p = 0.4826). These results suggest that the solution effect was equally strong regardless of the contact time.

**HGFIB:** All test series reduced the total cell number depending on the MR contact time (Figure 1B). The most cells were counted in the OCT test series after 1 min. The ranking for 1 min contact time was as follows: OCT > BET > MER > CHX > LIS. Slightly reduced cell numbers were measured after a contact time of 5 min, and the ranking corresponded to the ranking after 1 min contact time: OCT > BET > MER > CHX > LIS. The most HGFIBs were counted in the BET group after 15 min. The following ranking was observed: BET > OCT > MER > LIS > CHX. A longer contact time with OCT negatively influenced the cell number. A significantly lower influence of OCT compared to CHX, MER, and LIS at the contact times of 1 and 5 min was observed in HGFIBs (p < 0.005). No significant differences were observed after 15 min and compared to BET (p > 0.005) (Table 3).

**HNEPC:** All test series reduced the total cell number depending on the MR contact time (Figure 2B). The following ranking for 1 min contact time was observed: OCT > BET > CHX > LIS > MER. The most cells were counted in the BET group after a contact time of 5 min: BET > OCT > CHX > MER > LIS. A further reduction of the number of epithelial cells was observed at the 15-min contact time: OCT > BET > MER > LIS > CHX. The epithelial cell number showed no differences at the contact times of 1 min and 5 min between OCT and the other MRs (p > 0.005). A higher epithelial cell number was observed after 15 min of treatment with OCT compared to CHX (p < 0.005) (Table 3).

**Cell viability**

Cell viability was significantly reduced (p < 0.0001) after MR treatment (CHX, MER, LIS, BET, and OCT) in all test series (1, 5, and 15 min) compared to the controls (overall mean ± SE for control HGFIB: 95.5 ± 0.3%; HNEPC: 96.2 ± 0.3%). The variance analyses showed a significant influence of the MR used and the contact time, and a significant interaction of the MR and the contact time on HGFIB and HNEPC viability (p < 0.001).

**HGFIB:** A reduction of viability depending on MR contact time was observed in all test series (Figure 1C). The highest viability was measured in the OCT test series after a contact time of 1 min. The lowest cytotoxicity rankings were as follows: CHX < BET = LIS < OCT = MER (after 1 min), CHX < BET < LIS = MER < OCT (at the 5-min contact time) and at the longest contact time: CHX = BET < OCT < LIS < MER. A slight increase in cell activity was observed in the OCT group after a contact time of 15 min (Table 2).

### Table 3. Effects of mouth rinses on the cell numbers of HGFIB and HNEPCs and dependence on contact times of 1, 5, and 15 min.

| MR          | 1 min   | 5 min   | 15 min  | 1 min   | 5 min   | 15 min  |
|-------------|---------|---------|---------|---------|---------|---------|
| Control     | 6.7 ± 0.4 | 6.5 ± 0.4 | 6.4 ± 0.2 | 5.7 ± 0.2 | 5.4 ± 0.2 | 5.3 ± 0.2 |
| CHX 0.2%    | 4.1 ± 0.1 | 3.9 ± 0.1 | 3.4 ± 0.1 | 3.8 ± 0.1 | 3.9 ± 0.1 | 3.4 ± 0.1 |
| Meridol     | 4.2 ± 0.1 | 3.9 ± 0.1 | 3.7 ± 0.1 | 3.7 ± 0.1 | 3.7 ± 0.1 | 3.7 ± 0.1 |
| Listerine   | 3.8 ± 0.1 | 3.8 ± 0.1 | 3.6 ± 0.1 | 3.8 ± 0.1 | 3.7 ± 0.1 | 3.6 ± 0.1 |
| Betaisodona | 4.6 ± 0.1 | 4.5 ± 0.1 | 4.2 ± 0.1 | 4.2 ± 0.1 | 4.0 ± 0.1 | 3.7 ± 0.1 |
| Octenidol   | 4.8 ± 0.1 | 4.6 ± 0.1 | 3.9 ± 0.1 | 4.2 ± 0.2 | 3.9 ± 0.1 | 3.8 ± 0.1 |

### Table 4. Effects of mouth rinses on the viability of HGFIBs and HNEPCs and dependence on different contact times of 1, 5, and 15 min.

| MR          | HGFIBs (%) | HNEPCs (%) |
|-------------|------------|------------|
| Control     | 96.1 ± 0.5 | 96.2 ± 0.6 | 95.7 ± 0.6 |
| CHX 0.2%    | 76.1 ± 0.8 | 75.4 ± 1.3 | 72.5 ± 2.1 |
| Meridol     | 76.1 ± 0.8 | 63.7 ± 0.8 | 68.6 ± 0.8 |
| Listerine   | 75.9 ± 0.8 | 62.5 ± 1.5 | 68.6 ± 0.8 |
| Betaisodona | 79.9 ± 1.5 | 70.7 ± 0.5 | 72.2 ± 1.3 |
| Octenidol   | 80.0 ± 1.6 | 68.3 ± 1.6 | 75.9 ± 1.3 |
The cell diameter was significantly reduced \((p < 0.0001)\) after MR treatment (CHX, MER, LIS, BET, and OCT) in all test series (1, 5, and 15 min) compared to the control cells (HGFIB: 19.92 ± 0.09 micron; HNEPC: 21.3 ± 0.16 micron).

Variance analyses revealed a significant influence of the MR used and the contact time, and a significant interaction of the MR and the contact time on the diameter of the HGFIBs and HNEPCs \((p < 0.001)\).

HGFIB: The reduction in cell diameter in all test series depended on the MR contact time (Figure 1D). The largest diameter measured in the OCT test series after 1 min of contact time. The smallest cell diameter was measured in CHX treatment. The following ranking for 1 min contact time was observed: OCT > BET > MER = CHX > LIS (Table 4). The ranking changed after a contact time of 5 min because of a significant reduction of viability in the MR test series: OCT > BET > CHX > MER > LIS (Table 4). The highest viability was observed in the BET group after 15 min. The following ranking was observed: BET > OCT > LIS > MER > CHX (Table 4). The largest cell diameter was measured in the OCT test series after 1 min of contact time. The smallest cell diameter was measured in CHX treatment. The following ranking for 1 min contact time was observed: OCT > BET > MER > CHX = LIS (Table 5). The ranking changed slightly after a contact time of 5 min: OCT = BET > LIS > MER > CHX (Table 5). The largest cell diameter was measured for the BET group after 15 min: BET > OCT > LIS > MER > CHX (Table 5). A significantly lower influence of OCT and BET was observed on HGFIB diameter compared to CHX, MER, and LIS at all contact times \((p < 0.005)\). There were no significant differences between OCT and BET \((p > 0.005)\).

HNEPC: All test series, except MER, showed a reduction of the cell diameter depending on the MR contact time (Figure 2D). The following ranking for a contact time of 1 min was observed: CHX > MER > BET > OCT > LIS (Table 5). The following ranking was observed at a contact time 5 min: CHX > MER > OCT > BET > LIS. The cell diameter decreased for CHX at a contact time of 15 min, but it increased for MER. The following ranking was observed: MER > CHX > OCT > BET > LIS (Table 5). LIS showed the strongest influence on cell diameter compared to the other MRs at all contact times. MER showed a significantly lower influence on cell diameter compared to OCT, BET, and LIS at all contact times, and after 15 min compared to CHX \((p < 0.005)\). CHX showed a lower influence on cell diameter than OCT, BET, and LIS at 1- and 5-min contact times \((p < 0.005)\).

**Discussion**

This experimental, controlled, six-arm *in vitro* study of primary human cell lines investigated the cytotoxicity of various antiseptic MRs, with a particular focus on OCT. To the authors’ knowledge, there is only one study that investigated the biocompatibility and related cytotoxicity of MRs (Müller & Kramer, 2008), and no other results are currently available. The present investigation differentiated and investigated several different parameters of cytotoxicity. Some of the parameters, such as the cell diameter, were not described previously in the literature. Overall, the available data on cytotoxicity are insufficient.

The specific effectiveness of MRs on oral pathogens was not the subject of this investigation. Therefore, the possibility for a holistic evaluation and determination of the biocompatibility index based on the data of this study is limited.

The cytotoxic effects of all MRs on HGFIBs and HNEPCs were determined compared to control cells. The cytotoxic effects depended on the MR contact time, which was established for each of the investigated parameters (cell metabolism, cell number, cell viability, and cell diameter). A stronger negative influence on the investigated parameters was observed with longer MR contact times.

Relevant differences in cell metabolism between the solutions were not observed for the HGFIBs or HNEPCs. However, OCT and BET had the lowest influence on HGFIB

### Table 5. Effects of mouth rinses on the cell diameters of HGFIBs and HNEPCs and dependence on different contact times of 1, 5, and 15 min.

|   | HGFIBs (micron) |   | HNEPCs (micron) |
|---|----------------|---|----------------|
|   | 1 min          | 5 min| 15 min        | 1 min          | 5 min | 15 min        |
| Control | 19.8 ± 0.1 | 19.9 ± 0.2 | 20.1 ± 0.2 | 21.0 ± 0.2 | 21.6 ± 0.3 | 21.4 ± 0.4 |
| CHX 0.2% | 16.8 ± 0.2 | 16.4 ± 0.2 | 15.8 ± 0.1 | 20.8 ± 0.4 | 20.9 ± 0.6 | 19.1 ± 0.4 |
| Meridol | 17.0 ± 0.1 | 16.5 ± 0.1 | 15.9 ± 0.1 | 20.2 ± 0.2 | 20.2 ± 0.1 | 20.6 ± 0.2 |
| Listerine | 16.8 ± 0.1 | 16.6 ± 0.1 | 16.0 ± 0.1 | 18.3 ± 0.2 | 17.8 ± 0.3 | 17.3 ± 0.4 |
| Betaisodona | 17.8 ± 0.1 | 17.6 ± 0.1 | 17.1 ± 0.1 | 19.2 ± 0.1 | 18.7 ± 0.1 | 17.8 ± 0.1 |
| Octenidol | 18.0 ± 0.1 | 17.6 ± 0.1 | 16.9 ± 0.1 | 19.1 ± 0.1 | 18.8 ± 0.1 | 18.1 ± 0.1 |
metabolism. In HNEPCs, OCT also achieved results which were comparable to the established MR ($p > 0.005$).

Furthermore, OCT and BET showed the best results on cell numbers of HGFIBs and HNEPCs at all contact times ($p > 0.005$). A significantly lower toxic influence on HNEPCs was observed for OCT after 15 min compared to CHX ($p < 0.005$).

OCT and BET showed the best results on the cell viability of HGFIBs at all contact times ($p > 0.005$). However, a similar cytotoxic influence was observed for both of these MRs. MER showed significantly better results on the cell viability of HNEPCs than OCT ($p < 0.005$). OCT showed a slight influence on the cell diameter of HGFIBs and HNEPCs that depended on the concentration and time.

CHX is the gold standard of all MR ingredients. The most important advantage of CHX is the very high level of substantivity, which leads to a prolonged adherence of the antiseptic on hard and soft oral tissue. Therefore, the antiseptic is gradually released at an effective dose, which assures the persistence of its antimicrobial effect (Cousido et al., 2010). Nonetheless, negative effects were observed in many studies (Cabral & Fernandes, 2007; Eick et al., 2011; Park et al., 2014). Eick et al. demonstrated that commercially available CHX MRs have a very strong cytotoxic effect on the gingival fibroblasts in the MTT assay at different concentrations (0.01%, 0.06%, 1%, and 2%). The cells were exposed to the MR for 1 min and subsequently stored in a cell culture medium in this previous study (Eick et al., 2011). The results of the present investigation confirmed the cytotoxic influence of CHX and indicate a lower cytotoxic potential of OCT in the applied concentration compared to CHX. Significant differences in epithelial cell number reductions were observed between CHX and OCT. Therefore, the working hypothesis that OCT has a lower cytotoxic potential than CHX was partially confirmed. However, the differences between the different MR, investigated in this study, were minor.

The cytotoxic effect of OCT was often comparable to BET. Müller and Kramer found that OCT had the best biocompatibility index in HGFIBs in a comparison of several antiseptics, including OCT, CHX, and BET. The biocompatibility index helps provide objective evidence for the ratio between effectiveness and biological compatibility (Müller & Kramer, 2008), which is particularly important for antiseptics that are well tolerated and show low cytotoxic effects, such as BET. These antiseptics must be applied in significantly higher concentrations to achieve a sufficient antibacterial effect, and the cytotoxic effects are intensified. The cytotoxic effects of BET on fibroblasts and keratinocytes were described previously but not quantified (Sato et al., 2014). BET in MR is used in concentrations between $1.9 \times 10^3$ and $3.7 \times 10^3$ μM. Sato et al. used a significantly lower concentration in their study ($10^{-2}$ μM), but the contact time was much longer (1–2 d). The oral mucosa of rats showed epithelial damage at low concentrations of BET in their study (Sato et al., 2014). Furthermore, BET must be mixed in the applicable ratio because it is not commercially available as a ready-to-use solution. Therefore, the application is more complicated than OCT and other commercial MRs.

MER showed significantly better results than OCT on HNEPC viability ($p < 0.005$). MER has only been investigated previously in the framework of a clinical and histomorphometric animal study on rats to investigate the effect on epithelial cells. Excision wounds on the palate were treated with different antiseptics (1% CHX gel, LIS, MER), and the influence on the healing process was observed. None of the antimicrobial agents showed negative effects on the wound healing. The best results were achieved with a 1% CHX gel and LIS (Kozlovsky et al., 2007). Eick et al. observed a strong viability reduction when MER and LIS were applied to gingival fibroblasts in the MTT assay (Eick et al., 2011).

**Conclusion**

In summary, within the limitations of this study and the current literature, OCT can be recommended as an alternative to CHX because of its lower cytotoxic potential. However, the present study did not investigate the extent of a similar antiseptic effect. Therefore, further investigations are needed to verify whether OCT truly represents an alternative to CHX in clinical practice. It needs to be mentioned that the cytotoxic effect of OCT was often comparable to BET, which showed small cytotoxic effects.

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**Declaration of interest**

The authors declare that they have no conflict of interests.

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Supplementary material available online