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RESEARCH ARTICLE

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Effects of octenidine mouth rinse on apoptosis and necrosis of human fibroblasts and epithelial cells – an *in vitro* study

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ABSTRACT

This study aimed at comparing the cytotoxicity of a new octenidine mouth rinse (MR) on gingival fibroblasts and epithelial cells using different established MRs. Octenidol (OCT), Chlorhexidine 0.2% (CHX), Meridol (MER), Oral B (OB), and control (PBS only) were used. Human primary gingival fibroblasts (HGFIBs) and human primary nasal epithelial cells (HNEPCs) were cultivated in cell-specific media (2×10^5 cells/well) and treated with a MR or PBS for 1, 5, and 15 min. All tests were performed in duplicate and repeated 12 times. The apoptosis and necrosis were determined using a Caspase-3/7 assay and LDH assay, respectively. The data were analyzed using two-way analysis of variance with subsequent Mann–Whitney *U*-test. No significant differences could be found between the incubation times of the MR, neither for apoptosis nor necrosis ($p > 0.05$). Regarding apoptosis of HGFIBs, MRs had no influence at all. In HNEPCs, OCT induced relevantly lower apoptosis than CHX ($p = 0.01$). Considering necrosis, MER showed the lowest numbers of necrotic HGFIBs and HNEPCs, whereas OB induced the highest number of necrotic cells. The differences between both MR were statistically relevant ($p < 0.01$). OCT did neither differ from the other MRs nor from the control (PBS) in induction of necrosis in both cell types. In conclusion, the slightly negative effect of OCT considering apoptosis and necrosis of HGFIBs and HNEPCs is nearly the same or even lower compared to the established MRs included in this study. The results confirm that OCT is a potential alternative to CHX.

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Mouth rinse; octenidine; apoptosis; necrosis; human fibroblasts; human epithelial cells

Introduction

Antimicrobial mouth rinses (MRs) play a role in the antiseptic therapy of the oral cavity, thus supporting the oral biofilm management in selected indications. Evidences are emerging which suggest the new antimicrobial mouth rinse (MR) octenidine dihydrochloride, trade name Octenidol (OCT), as a potential alternative to commonly used MRs, such as Chlorhexidine (CHX), amine and stannous fluorides as well as cetylpyridinium chloride (CPC) (Müller and Kramer 2008, Schmidt *et al.* 2015, Welk *et al.* 2015).

CHX 0.2% can still be considered the gold standard MR in the oral antiseptic therapy (Quirynen *et al.* 2005, Giannelli *et al.* 2008). It has been extensively used and tested concerning its effect on bacteria, its substantivity in the oral cavity, and its usage within the field of periodontal therapy (Löe and Schiott 1970, Quirynen *et al.* 1995, Cronan *et al.* 2006, Cousido *et al.* 2010). However, adverse effects have also been described for CHX regarding the cytotoxic effects on periodontal cells (Cabral and Fernandes 2007, Eick *et al.* 2011, Millhouse *et al.* 2014, Park *et al.* 2014), periodontal healing, and regeneration (Alleyn *et al.* 1991, Mariotti and Rumpf 1999).

Therefore, MRs with lower cytotoxic potential but comparable or higher antibacterial effect are deemed to be desirable. It has yet to be determined how far these expectations can be fulfilled by OCT. In a prior study, our working group could show favorable results for OCT concerning the cell metabolism, cell number, and viability of fibroblasts and epithelial cells (Schmidt *et al.* 2015). The antibacterial effect of OCT was found to be similar or even higher than that of CHX (Müller and Kramer 2008, Latimer *et al.* 2015, Welk *et al.* 2015) with a higher biocompatibility of OCT considering fibroblast cytotoxicity (Müller and Kramer 2008). However, OCT should not only be compared to CHX but to other alternative MRs available for antiseptic therapy as well.

One of these alternative MRs is Meridol (MER), an antiseptic based on the active ingredients amine fluoride and stannous fluoride (AFSF). The antibacterial and plaque-reducing properties against *in situ* biofilm did not differ between MER and CHX according to a study by Auschill *et al.* (2005). Eick *et al.* demonstrated pronounced cytotoxic effects of MER on gingival fibroblasts, which were similar to those of CHX (Eick *et al.* 2011). Recently, Balloni *et al.* showed that AFSF mouth rinse does not induce or induces to a lesser extent the onset

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

Designation	Mouth rinses	Active ingredient	Manufacturer
CHX	Chlorhexidine digluconate, ready-to-use solution 0.2%	Chlorhexidine (digluconate)	Engelhard Arzneimittel GmbH & Co.KG, Niederdorfelden, Germany
MER	Meridol	Amine fluoride and stannous fluoride (AFSF) (AmF/SnF_2)	GABA GmbH, Lörrach, Germany
OB	Oral B	Cetylpyridinium chloride (CPC), natrium fluoride (NaF)	Procter and Gamble GmbH, Schwalbach am Taunus, Germany
OCT	Octenidol	Octenidine dihydrochloride	Schülke GmbH, Norderstedt, Germany

of irritation and/or cytotoxicity compared to CHX (2016). This result could be confirmed by a prior study of our group. However, we found favorable results for the cytotoxic (less cytotoxic) effect of MER on epithelial cells compared to CHX, OCT, Betaisodona, and Listerine (Schmidt *et al.* 2015).

For CPC, Latimer *et al.* showed a significant antibacterial efficacy against *Fusobacterium nucleatum*, an oral bacterium associated with biofilm formation and gingival disease (Latimer *et al.* 2015). Slightly less cytotoxic effects on murine fibroblasts are described for CPC in comparison with CHX (Müller and Kramer 2008). Zheng *et al.* even suggested a prevention of bone loss due to the inhibition of the osteoclast differentiation by CPC (Zheng *et al.* 2013). Therefore, CPC is an active ingredient worth to be taken into consideration, which is commercially available as a ready-to-use solution in different concentrations provided by different manufacturers.

The current investigation is an addition to our prior study and aims at evaluating the cytotoxicity of OCT in comparison with commercially available ready-to-use MRs based on CHX (0.2%), stannous fluoride, and CPC. Releases of extracellular lactate dehydrogenase (LDH) and Caspase-3/7 were used as indicators for the necrosis and apoptosis of fibroblasts and epithelial cells, respectively.

Materials and methods

The present investigation was an experimental, controlled, five-arm *in vitro* study of primary human cell lines with the aim to investigate the cytotoxicity of several antiseptic mouth rinses while differentiating between apoptosis and necrosis.

Materials

Cells

Cryoconserved primary human gingival fibroblasts (HGFIBs; order number 1210412, Provitro GmbH, Berlin, Germany) and primary human nasal epithelial cells (HNEPCs; order number 1210711, Provitro GmbH, Berlin, Germany) were used as basic material for cell cultivation. These primary human cells were cultivated, frozen, and thawed under the same conditions and as described before (Schmidt *et al.* 2015).

Mouth rinses (MRs)

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), and CPC (OB: Oral B, Procter and Gamble GmbH, Schwalbach am Taunus, 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). CHX, MER, OB, and OCT were sterile-filtered and used without dilution in subsequent cell cultures. The preparations used in this study were commercially available mouth rinses. None of them contained any ethanol. The control groups (CTR) were treated with PBS (phosphate-buffered saline) without the addition of antiseptics.

After cell counting, the cells were transferred to 96-well plates according to the respective group and contact time. The cell concentration was adjusted to 2×10^5 cells/100 µl culture medium per well and incubated at a constant nitrogen content of 5%, at a temperature of +37 °C, and under saturated humidity within a CO_2 incubator. The medium was exchanged after 24 hours in order to remove non-adherent cells and supply nutrients to the cells. The cells were checked light microscopically after each step. After reaching a 75% confluence, culture medium was removed and the cells were treated with 100 µL of the respective oral antiseptic (Table 1). All tests were performed in duplicate and repeated 12 times. Each solution remained on the cells for 1, 5, or 15 minutes, respectively, at a constant CO_2 content of 5%, at a temperature of +37 °C, and under saturated humidity within a CO_2 incubator. Active ingredient solutions were aspirated. The wells were rinsed with 100 µL PBS for 30 seconds and refilled with 100 µL of fresh culture medium. In pretests, the release of Caspase-3/7 was found to be at a maximum level 24 hours after MR application. Therefore, the cells were cultivated for another 24 hours at a constant nitrogen content of 5%, at a temperature of +37 °C, and under saturated humidity within a CO_2 incubator. Finally, a Caspase-3/7 assay and a CytoTox-ONE™ Homogeneous Membrane Integrity Assay (LDH assay) were performed.

Detection of Caspase-3/7 activity in cell culture

One hundred microliter of Apo-ONE® Caspase-3/7 reagent (substrate and buffer) was added to each well, and the initial fluorescence of each well was measured (excitation wavelength of 498; emission wavelength: 521 nm; determined empirically). The contents of the wells were gently mixed using a plate shaker at 300 rpm from 30 seconds up to read time. After incubation of the plates at room temperature for 30 minutes, the fluorescence of each well was measured (excitation wavelength of 498; emission wavelength: 521 nm; determined empirically).

Detection of LDH in cell culture supernatants

The 96-well assay plates for the LDH assays were removed from the 37 °C incubator and equilibrated to 22 °C for

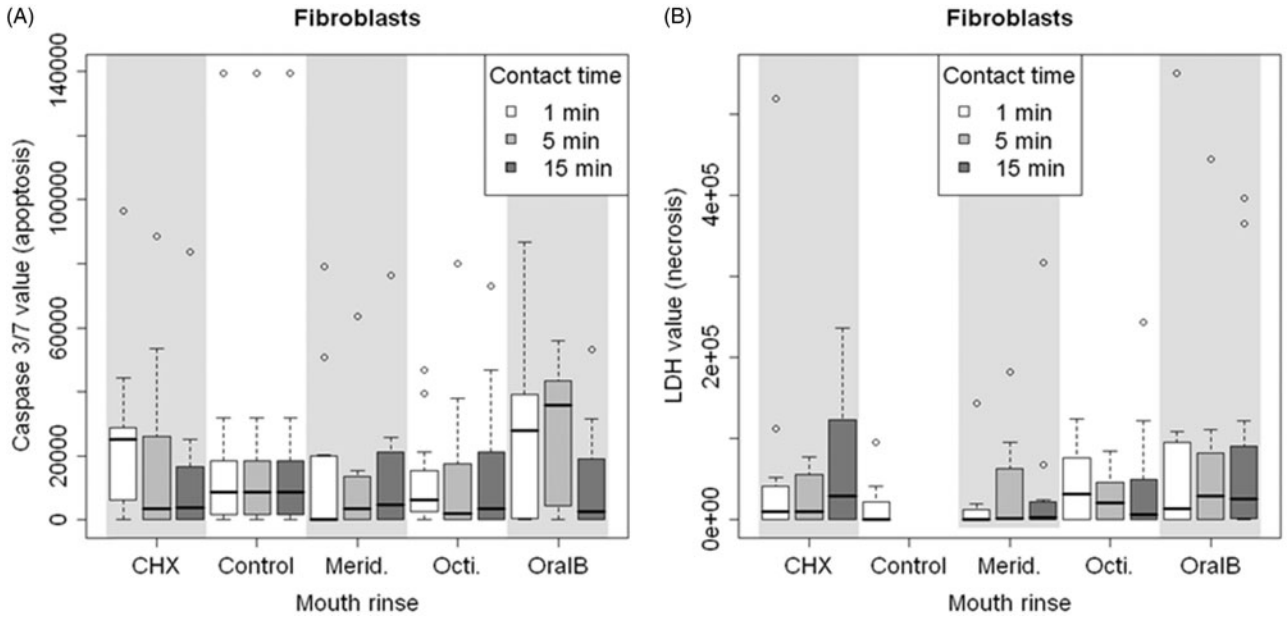

Figure 1. Results of the Caspase-3/7 assay (A) and LDH assay (B) of HGFIBs after incubation with several mouth rinses for different times (1, 5, and 15 minutes). Merid: Meridol; Octi: Octenidol.

approximately 20–30 minutes. 100 µL of CytoTox-ONE™ reagent was added to each well and mixed for 30 seconds. After an incubation period at 22 °C for 10 minutes, 50 µL of Stop Solution was added to each well in the same order of addition that was used for adding the CytoTox-ONE™ reagent. The plate was shaken for 10 seconds and the fluorescence was recorded (excitation wavelength: 560 nm; emission wavelength: 590 nm; as recommended).

Statistical analysis

The influence of the different mouth rinse solutions and the contact times and the interaction of these two factors on the measurements were investigated separately according to the cell types and methods of measurement using a non-parametric two-way analysis of variance (ANOVA) (Brunner *et al.* 2002) plus interaction term. In case of a significant main effect, the results of the mouth rinse solutions were pairwisely compared to the control data and to each other using the Mann–Whitney *U*-test.

The significance level was set to $\alpha = 5\%$. A significant difference for the paired comparisons was only assumed when the p value was below the Bonferroni-adjusted significance level of $0.05/10 = 0.005$. All testing procedures and figure constructions were conducted using the software R (version 2.8, www.r-project.org).

Results

Apoptosis (Caspase-3/7)

HGFIBs: In conclusion with the two-way ANOVA (Supplementary Table 1), the number of apoptotic cells in the HGFIBs after the treatment with the different MRs (CHX, MER, OB, and OCT) was not influenced in all test series (1, 5, and 15 minutes) (each $p > 0.05$, Figure 1(A), Table 2) compared to the control cells (median/range for control: 8758/0–139600, Table 2). The ranking for the apoptotic HGFIBs after the treatment with the MR was OB > CHX > OCT > MER (Table 2, Figure 1(A)). The interaction between MR and contact time had no significant influence as well (Supplementary Table 1).

Table 2. Descriptive statistics are given as the median (range) and mean ± standard deviation (SD) of the measurements for caspase and LDH of HGFIBs and HNEPCs after treatment with control and oral antiseptics (MRs).

			Median (range)
HGFIBs	Caspase	CTR	8758 (0–139600)
		CHX	8586 (0–96380)
		MER	2568 (0–79080)
		OCT	5736 (0–79900)
		OB	18880 (0–86580)
	LDH	CTR	0 (0–95850)
		CHX	20640 (0–519100)
		MER	0 (0–317800)
		OCT	11810 (0–243200)
		OB	27630 (0–551600)
HNEPCs	Caspase	CTR	0 (0–85450)
		CHX	0 (0–94790)
		MER	0 (0–64470)
		OCT	0 (0–54150)
		OB	0 (0–65120)
	LDH	CTR	0 (0–69000)
		CHX	0 (0–158400)
		MER	0 (0–251600)
		OCT	0 (0–270400)
		OB	25280 (0–323300)

HNEPC: Neither contact time nor interaction between MR and contact time showed a significant influence on the apoptosis of the HNEPCs in two-way ANOVA ($p > 0.05$, Supplementary Table 1). Overall, only small differences were found regarding the influence of the different MRs ($p = 0.03$, Supplementary Tables 1 and 2). Except for the differences between CHX vs. MER ($p < 0.01$) and CHX vs. OCT ($p = 0.01$), no statistical significances were found ($p > 0.05$). Due to the Bonferroni adjustment, the differences between CHX vs. MER and CHX vs. OCT cannot be called significant, even though they are still showing a trend. The ranking for HNEPC apoptosis after the treatment with the MRs was CHX > OB > MER > OCT (Table 2, Figure 1(B)).

Necrosis (LDH)

ANOVA showed that necrosis was neither influenced by contact time nor by interaction between the MRs and the contact time in HGFIBs and HNEPCs, respectively.

HGFIBs: Two-way ANOVA showed that the MR significantly influenced necrosis of HGFIBs ($p < 0.05$, Supplementary Table 1). The ranking for the necrotic fibroblasts after the treatment with the MRs was OB > CHX > OCT > MER (Table 2, Figure 2(A)), with the differences between CHX vs. MER ($p = 0.04$) and MER vs. OB ($p < 0.05$) being of statistical relevance without being significant after the Bonferroni adaptation The other comparisons between the MRs and CTR did not show relevant differences considering their influence on necrosis of HGFIBs ($p > 0.05$).

HNEPCs: In ANOVA, a significant influence of the MR was found considering necrosis of HNEPCs ($p = 0.03$, Supplementary Table 1). The ranking for the necrotic HNEPCs was OB > OCT > CHX > MER. MER showed less cytotoxic effects regarding the necrosis of HNEPCs with a statistically significant difference compared to OB ($p < 0.01$). OCT and CHX had a similar effect on the necrosis of the HNEPCs ($p = 0.87$). Compared to the CTR group, after the treatment with OB, a significant higher number of necrotic HNEPCs were found ($p < 0.01$). The effect of the other MRs did no differ from the CTR ($p > 0.05$).

Discussion

This experimental, controlled, five-arm *in vitro* study of primary human cell lines evaluated the cytotoxicity of OCT in comparison with the commercially available ready-to-use MRs CHX, MER (AFSF), and OB (CPC). Cytotoxic effects were demonstrated by the release of extracellular LDH and Caspase-3/7, indicating either necrosis or apoptosis of fibroblasts and epithelial cells. The current study adds further information to our previously published study, which investigated the cytotoxicity of MRs in the same primary human cell lines (Schmidt *et al.* 2015). In contrast to this prior investigation, the CPC-based MR OB was used instead of Betaisodona (BET) due to the fact that BET is not commercially available as a ready-to-use solution.

The results of this study showed that the necrosis of HGFIBs and HNEPCs and the apoptosis of HNEPCs cells were influenced by the MR but not by the contact time. The shortest contact time was chosen to be 1 minute because patients rinse for 30 seconds to one minute in daily clinical practice. Beneath this short time effect, apoptosis and necrosis after overexposure (incubation times of 5 minutes and 15 minutes) were considered to evaluate whether there are effects at all and if they depend upon the contact time. Furthermore, longer contact times should be determined because ingredients stay longer than just for the duration of the mouth rinse due to substantivity, especially in case of CHX. Contact times were chosen in concordance with the prior study of Schmidt *et al.* (2015). Other authors described incubation times of more than one minute as well (Alleyn *et al.* 1991, Park *et al.* 2014, Chen *et al.* 2016). As the results show, no influence of contact time was found.

In HNEPCs, the lowest rate of apoptotic cells was observed in the OCT group, whereas the CHX group showed the highest rate. Considering induction of necrosis, MER showed the most favorable results in both cell lines without any significant difference in comparison with OCT. OB induced the highest rate of necrosis.

The antibacterial effect of the MRs on oral pathogens was not investigated in this study. Thus, the options for a holistic evaluation and the determination of the biocompatibility index are limited. However, each of the MRs included in this study has been proven to have an adequate antibacterial efficacy (Cousido *et al.* 2010, Feres *et al.* 2010, Otten *et al.* 2010, Latimer *et al.* 2015, Mutters *et al.* 2015, Welk *et al.* 2015, Decker *et al.* 2016). Furthermore, Müller and Kramer found that within a comparison of several antiseptics, OCT had the

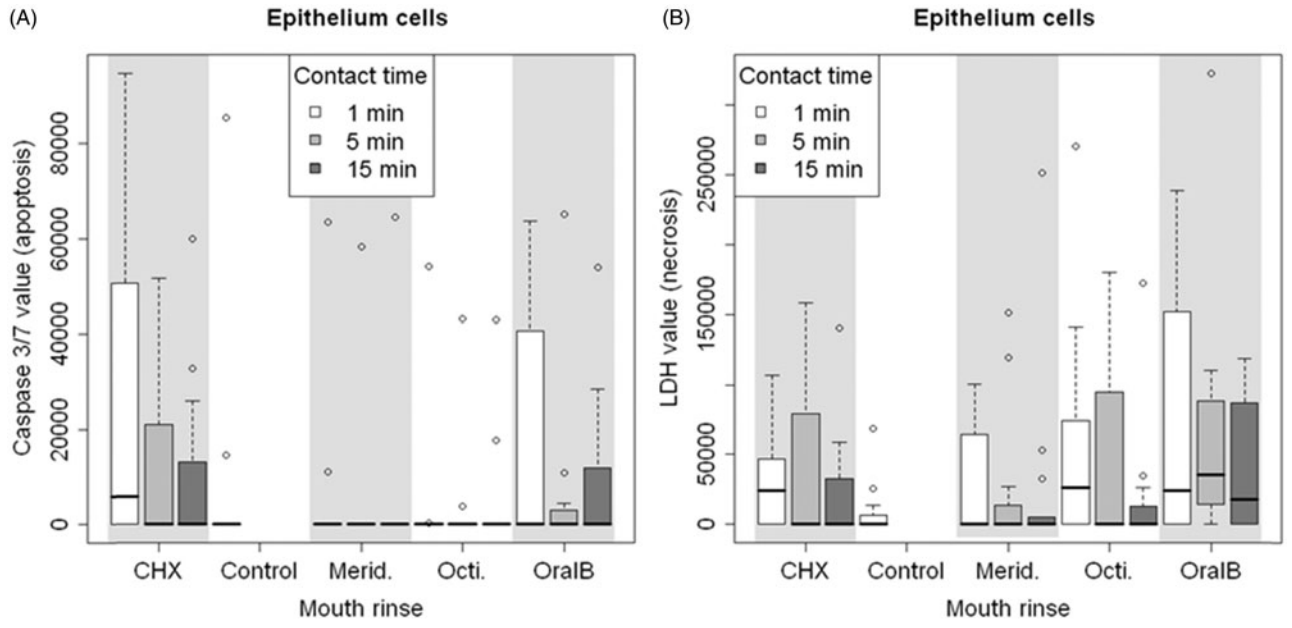

Figure 2. Results of the Caspase-3/7 assay (A) and LDH assay (B) of HNEPCs after incubation with several mouth rinses for different times (1, 5, and 15 minutes). Merid: Meridol; Octi: Octenidol.

best biocompatibility index (BI) in murine fibroblasts regarding the antibacterial efficacy against *E. coli* and *S. aureus*. The BI helps to provide objective evidence for the ratio between effectiveness and biological compatibility (Müller and Kramer 2008).

However, there is a lack of evidence concerning the cytotoxic effects of different MRs on periodontal cells such as fibroblasts and epithelial cells. Considering the mechanism of cell death after the application of healthcare ingredients, only a few studies could be found which were in relation to CHX (Giannelli *et al.* 2008, Faria *et al.* 2009) and CPC (Hagi-Pavli *et al.* 2014). Therefore, this investigation was performed as an addition to a prior study of this working group which showed that OCT can be recommended as an alternative to CHX due to its lower cytotoxic potential. The current investigation aims at specifying the mechanism of cell death induced by OCT in comparison with different commercially available ready-to-use MRs. To the authors' best knowledge, this is the first study to evaluate OCT in comparison with the established MRs MER, CHX (0.2%), and OB concerning apoptosis and necrosis. Since only few data are available for the apoptosis and necrosis of periodontal cells after the application of MRs, options are limited for discussing and comparing the data obtained in the present investigation.

In accordance with a previous study, the results of this study confirm that CHX stimulates apoptosis and necrotic cell death in epithelial cells as well as in fibroblasts (Giannelli *et al.* 2008). CHX is supposed to be cytotoxic because of the stress for the endoplasmatic reticulum which results in the release of calcium ions and reactive oxygen species, which are mediators for mitochondrial dysfunctions and the main reason for apoptosis and necrosis (Giannelli *et al.* 2008, Faria *et al.* 2009). However, in HGFIBs, a higher cytotoxicity of CHX could be shown compared to treatment of cells with pure water, whereas a significant higher level of apoptosis of cells was found in the pure water group (John *et al.* 2014). In the present study, no influence of any MR was found for apoptosis of fibroblasts compared to treatment with CTR. John *et al.* propose that the low results of apoptosis in the CHX group were due to the cytotoxic action of CHX.

OB showed an induction of necrosis (release of LDH) comparable to CHX in HGFIBs, and an even higher induction of LDH release in HNEPCs than the other MRs. The results of Feres *et al.*, who found CPC (0.05%), which is the active ingredient of OB, to be less cytotoxic than CHX (0.12%), could not be confirmed (Feres *et al.* 2010). OCT showed less induction of apoptosis than the other MRs in HNEPCs. These findings could be an explanation for the good results of OCT considering the cell number of HNEPCs (Schmidt *et al.* 2015). The lower induction of a LDH release by MER in HNEPCs and HGFIBs, which did not significantly differ from OCT, may be associated with the higher viability of the cells treated with OCT and MER (Schmidt *et al.* 2015). In contrast, Eick *et al.* found a strong viability reduction when MER was applied to gingival fibroblasts in the MTT assay (Eick *et al.* 2011).

Considering possible hypotheses about the mechanisms that lead to necrosis or apoptosis, changes in extracellular matrix metabolism could be involved in induction of necrosis. Balloni *et al.* showed that a reduction in fibroblast and keratinocyte substrate adhesion capacities, induced by CHX, was associated with a higher rate of cell death (2016). Furthermore, inflammatory markers which are induced by stimulation with MRs (e.g., interleukin-8 and CXCL-1) could induce apoptosis by oral cells. Other authors hypothesized that cytotoxic action of a MR could be an explanation for low results of apoptosis (John *et al.* 2014).

In context with the known parameters of the cytotoxic effects of MRs (cell viability, cell number, cytotoxicity assay/MTT), it was shown that CHX is more cytotoxic than OCT (Müller and Kramer 2008, Schmidt *et al.* 2015). However, these differences are small and clinical relevance remains unclear. Müller and Kramer found that OCT had the best biocompatibility index and therefore the most favorable ratio between effectiveness and biological compatibility in fibroblasts in a comparison of several antiseptics, including OCT, CHX, and BET (Müller and Kramer 2008). The well-tolerated BET must be applied in significantly higher concentrations to achieve a sufficient antibacterial effect, thus intensifying the cytotoxic effects (Sato *et al.* 2014). Furthermore, BET is not available as a ready-to-use solution, which is what makes the application more complicated.

Strengths and limitations

To our best knowledge, this study is the first investigating necrosis and apoptosis induced by the MR OCT in comparison with well-established and relatively well-studied MRs. Furthermore, the influence of contact time and the interaction between MR and contact time considering the influence on apoptosis and necrosis were investigated. However, the study was performed in primary human cell lines (fibroblasts and nasal epithelial cells) grown in monolayers. Usage of HNEPCs as well as the monolayer model set some restrictions in evaluation of the study's results considering their transferability to the oral cavity. It would have been favorable to use oral (buccal) keratinocytes, ideally in a 3D model as described by Koschier *et al.* (2011). For a comprehensive investigation, release of inflammatory markers which simultaneously influence apoptosis (e.g., interleukin-8 and CXCL-1) by oral cells upon stimulation with MRs as well as expression of markers involved in cell metabolism (e.g., integrins, laminins; Balloni *et al.* 2016) and apoptosis (e.g., Annexin V) should be considered in further research.

Conclusions

In summary, we could confirm the results of the prior study of this working group implying that OCT can be recommended as an alternative to CHX when viewed in the context of cytotoxicity. The results provide further evidence that the cytotoxic potential of OCT is slightly lower than that of CHX and comparable to well-tolerated established MRs, such as MER and BET. The antiseptic efficacy of OCT is well documented in current literature and comparable or even higher than that of established MRs, including the gold standard CHX. More detailed *in vitro* studies investigating inflammatory markers as well as expression of markers involved in cell

metabolism and apoptosis, performed in 3D models of oral keratinocytes, are required to determine the cytotoxic effects of OCT more comprehensively. Furthermore, clinical studies are needed in order to verify whether OCT truly represents an alternative to CHX in clinical practice.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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