

Cytotoxic effects of three different oral antiseptic solutions on epithelial cells of buccal mucosa

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Abstract: A great number of oral antiseptic solutions are on the market with the target of reducing the number of bacteria, of having antiplaque, anti-inflammatory and anti-cariogenic effects or also of eliminating halitosis. Numerous studies testing the antimicrobial effect of antiseptic solutions found that all of them contained octenidine and chlorhexidine. There are few data on the cytotoxicity of antiseptics on keratinocytes of buccal mucosa. The aim of this study is to test and compare the cytotoxic effects of three different commercial oral antiseptic solutions on keratinocytes taken from smear of buccal mucosa. The solutions under study here are Hibidex DAP* 0.12%, Ozosept* and Octenisept* sprays. Smears were taken from buccal mucosa of six healthy volunteers. The samples were stained with acridine orange/ethidium bromide (AO/EB), and observed under a fluorescence microscope. The cytotoxic effect was expressed in percentage of cell viability. The cytotoxicity of the oral antiseptic solutions increases progressively with time. We noticed that the cytotoxic effects of some solutions manifested gradually, while the abrupt drop in cell viability had been verified when it came to Hibidex DAP*. The active ingredient of both Octenisept* and Ozosept* has a lower level of cytotoxicity compared to Hibidex DAP* on oral mucosal keratinocytes.

Keywords: oral antiseptics; oral keratinocytes; buccal mucosa; smear; cytotoxicity.

Introduction

Oral mucosa is constructed of loose connective tissue and non-keratinized stratified squamous epithelium. Superficial layer of this epithelium is formed of live, flat-shaped cells that have nuclei, organelles, diffuse keratin filaments and keratohyaline granules [1]. The epithelium is permeable to certain chemicals which can be utilized for therapeutic purposes, but is also the most exposed to the harmful effects of various substances that can be found in the oral cavity [2]. A number of different antiseptic solutions are used on the market as mouth rinses consisting typically of essential oils, alcohol, solvents, and as active substance chlorhexidine digluconate and octenidine [3,4]. In addition to their role in maintaining oral hygiene, oral antiseptics are applied in the treatment of various oral diseases such as gingivitis, periodontitis and various inflammatory conditions of the oral mucosa [5]. The effects of antiseptic solution are reflected in the reduction of the number of bacteria in the oral cavity, in having antiplaque, anti-

inflammatory and anti-cariogenic effect as well as in eliminating unpleasant breath [6,7]. The main characteristic of antiseptic solutions is their ability to destroy microorganisms or inhibit their growth after their local application.

Various studies examined the antimicrobial effect of different antiseptic solutions, showing that this effect is specifically evident in solutions containing octenidine and chlorhexidine [8-11]. Only few studies have analyzed the cytotoxic effect of antiseptic solution on living cells particularly on culture of epithelial cells, fibroblasts, osteoblasts, and endothelium of nasal mucosa [12-14]. The study of Flemingson et al. [15] who have examined the influence of antiseptic solutions on cultivated fibroblasts, showed that the most toxic antiseptics were the ones containing chlorhexidine. Giannelli et al. [12] showed that this negative effect is proportional to the concentration of the active substance and the time of exposure. A study of Schmidt et al. [14] showed that antiseptics reduce the metabolic activity of fibroblasts and epithelial cells as well. They stressed that after 15 minutes of

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DOI://<http://dx.doi.org>

chlorhexidine exposition fibroblast and epithelial cell viability dropped to 68% and 65% respectively. Three types of mouth rinses investigated in our have different composition and well known mode of antimicrobial action. Octenidin hydrochloride belongs to the class of bispyridinamines and was initially developed to be used as topical antimicrobial agent with a broad spectrum effective against both Gram negative and Gram positive organisms, as well as certain fungi, including *Candida albicans* [16]. Ozosept concentrated solution is used for regular hygiene of the oral cavity, maintaining the health of the gums, larynx and pharynx. Thanks to its special combination of ingredients (Thymol, benzoin acid, menthol, metilsalicilat, eosin, ethanol), it is recommended as a supplement to the treatment of gingivitis, periodontitis, mouth ulcers, stomatitis, tonsillitis, pharyngitis [17]. Chlorhexidine digluconate binds to the microorganisms surface thanks to his cationic group, affecting the integrity of the cell wall with a loss of important constituents of the cytoplasm due to the increased permeability. The retained chlorhexidine absorbed in the oral mucosa, is then gradually released in saliva, thus providing long-lasting bacteriostatic effect. Other studies reported its role in the induction of cell death (apoptosis/necrosis) through mitochondrial dysfunction, increase of the intracellular calcium and reduction of oxygen [14].

No studies to our knowledge have analyzed the effect of antiseptic solution on the oral epithelium cell smears. The aim of our study is to analyze and compare the cytotoxic effect of three different commercial mouth rinses Hibidex DAP, Ozosept and Ocetenisept on keratinocytes isolated from buccal mucosal smear.

Materials and Methods

Sample collection

Swabs were taken from the buccal mucosa with wooden spatula from six healthy volunteers (5 female, one male, aged 24-28) in order to collect surface epithelial cells. The volunteers were informed about the design of the study and gave their written consent. All procedures were approved by the Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac (No. 01-7501). All cells were mixed together and then divided in two groups: control group cells were placed in 0.9% saline solution in order to preserve their viability and the experimental group cells were further exposed to the three oral antiseptics.

Oral antiseptics

The solutions used in the research were: 0.12% Hibidex DAP* (Hibidex DAP, Galenika, Belgrade, Serbia), 1 ml solution contains 1.2 mg of 20% chlorhexidine digluconate, spray Ozosept* (Ozosept

– Pharmanova, Belgrade, Serbia) containing chamomile, benzoic acid, thymol, menthol, ethanol, essential oils of peppermint, anise and eucalyptus and spray Ocetenisept* (Ocetenisept - Schülke, Hamburg, Germany) with 0.1 g octenidine dichloride and 2 g phenoxy ethanol. Octenidin-dihydrochlorid is a cationic surface-active compound. It adheres to cell wall and membrane components of microbial cells and leads to the formation of non-cytotoxic complexes at the site of action with the final destruction of the cell functions. Another active substance is fenoxietanol and its antimicrobial activity is based on an increased permeability of potassium ions across the cell membrane. Ozosept active substances interacts with bacteria membranes affecting its permeability causing partial damage without the lyse of the membranes which prevents the release of endotoxin in the organism. It also inhibits the synthesis of the respiratory chain proteins, which blocks the cellular respiration leading to microorganisms death [18]. Chlorhexidine digluconate has a cytotoxic effect on epithelial cells of the gingiva, and reduces the proliferation of human fibroblasts and collagen protein production depending on the applied dose. Due to the presence of propylene glycol it may cause irritation of oral mucosa. In order to obtain 90% concentration of the oral antiseptics in the experimental group, 0.15 ml of single cell suspension was added to 1.35 ml of antiseptic solution. On the other hand, control group cells were kept in the saline solution during the whole experiment to assess their viability throughout the study together with the experimental group cell.

Cell viability and cytotoxicity

After the initial determination of the cell viability, cytotoxicity of the antiseptic solutions was measured after 1, 3, 5, 10, 15, 20, 25 and 30 min of treatment. Cells were stained with acridine orange/ethidium bromide (AO / EB) dye and observed under the fluorescence. Cells whose nuclei and cytoplasm during the experiment were stained with EB were considered dead, while cells stained only with AO (with intact cell membrane) were considered live.

“Transitional” cell form whose nuclei and cytoplasm were mostly stained with AO, and only parts of the cytoplasm stained with EB were considered still alive in our experiment. All measurements were performed in triplicate, and mean value is calculated as the final result. Since the cell counting is a time consuming process, in order to avoid the unwanted prolonged effect of antiseptic solution, viability assessment was carried out subsequently, using the photomicrographs. Images of stained cells were made with a digital camera mounted on a microscope Olympus BX 51. The cytotoxic effect is expressed as a percentage of live cells.

Results

The overall number of cells obtained by buccal smears was 126×10^3 . The cell viability in the control group was 100% and kept at the same level during the whole experiment. The evaluation of the cytotoxicity of the antiseptic solution Hibidex DAP* has showed that the viability of keratinocytes taken from buccal mucous membranes, progressively decreases in time (Table 1, Figure 1). After the first minute the percentage of viable cells has already decreased to 65%, after three minutes to 30%, while after five minutes viability was 0%. Testing the effect of solution Octenisept* has shown that the viability of the cells also decreases progressively during the time (Table 1). A significant difference is that the viability of the keratinocytes in this case was maintained at the initial 100% for whole ten minutes, whereas after fifteen minutes of Octenisept* treatment, the percentage of viable cells dropped to 65%, after twenty minutes to 30%, and after twenty five minutes there were no viable cells.

The cytotoxicity of Ozosept* has been also verified in our experiment, but it has been shown that it has the least impact on the viability of oral keratinocytes. Toxic effect of the Ozosept* solution on buccal keratinocytes begins to manifest after fifteen minutes whereby the viability drops to 85% and over the time of exposure the percentage of dead cells grows. After twenty five minutes viability was 70% and after 30 min there were no more viable cells (Table 1, Figure 1).

During the study it was observed that the cytotoxic effect of some solutions manifested gradually since we had verified cells on our preparations that indicated the presence of ethidium bromide in the cytoplasm while the nuclei and cytoplasm were dominantly stained with acridine orange which showed that cells were still viable (Figure 2). In our study, such cells were designated as “transitional” and classified as viable since the EB only partially entered the cytoplasm. These transitional forms were not verified when it came to the effect Hibidex DAP* because it showed strong cytotoxic effect in a very short period of time.

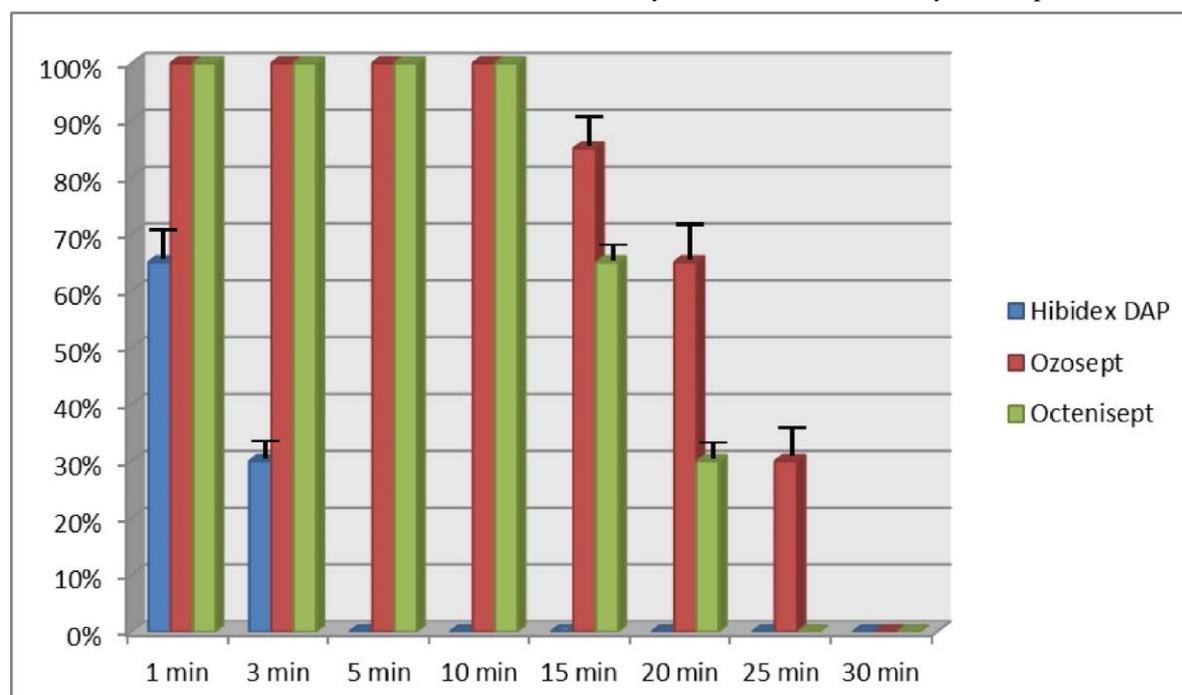


Figure 1. Decrease of viability of keratinocytes from smear of oral mucosa under the influence of antiseptic solutions over time (expressed in percentages of viable cells, mean \pm SD)

Table 1. Decrease of viability of keratinocytes from smear of oral mucosa under the influence of antiseptic solutions over time (expressed in percentages of viable cells).

| | 1 min | 3 min | 5 min | 10 min | 15 min | 20 min | 25 min | 30 min |
|--------------------|-------|-------|-------|--------|--------|--------|--------|--------|
| Hibidex DAP | 65% | 30% | 0% | 0% | 0% | 0% | 0% | 0% |
| Ozosept | 100% | 100% | 100% | 100% | 85% | 65% | 30% | 0% |
| Octenisept | 100% | 100% | 100% | 100% | 65% | 30% | 0% | 0% |

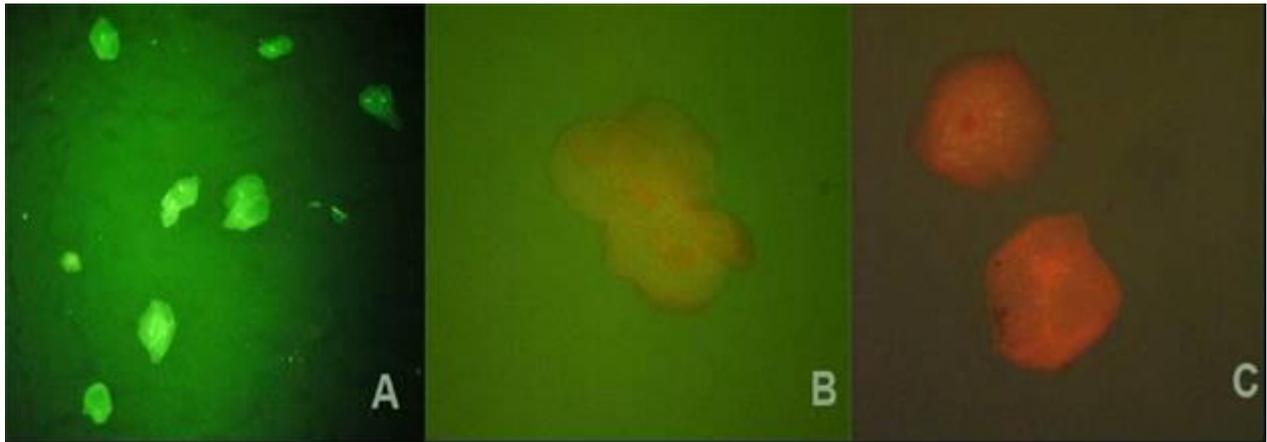


Figure 2. Viability of keratinocytes. Control keratinocytes (A). "transitional form" (B). nonviable keratinocytes (C) AO / EB staining, A (x100), B and C (x400)

Discussion

Antiseptic mouth-rinses containing active antimicrobial substances express positive clinical effects by reducing the number of bacteria in the oral cavity, have anti-inflammatory and anti-cariogenic influence and eliminate halitosis. However, they all show a negative, cytotoxic effect on the superficial keratinocytes of the oral mucosa [6-11]. As shown in our study, chlorhexidine digluconate has the strongest cytotoxic effect, leading to a significant drop in viability of keratinocytes isolated from smear of oral mucosa. After three minutes, the drop of keratinocytes viability was 70%, and after five minutes all treated keratinocytes were dead. Octenisept* and especially Ozosept* also have this negative effect but much less expressed. They cause 100% cytotoxicity just only after twenty five or thirty minutes.

These results are in correlation with the findings of Müller [8] and Goldschmidt [19] who found a similar effect of chlorhexidine, but on human fibroblasts and osteoblasts in culture. Because of the structure and biological behavior, as well as the natural environment in which fibroblasts and osteoblasts exist in human tissues are not nearly the same as those of keratinocytes of oral mucosa, this coincidence of the results should be taken with a grain of salt. It is interesting that there are not many scientific papers aimed to test cytotoxic effects of oral antiseptics, and only a few references are related to the effects of these solutions on oral keratinocytes in culture [13,20]. On the other hand, in the available literature we did not find studies involving cells obtained by smear of oral mucosa. Tsutsui et al. [20] showed that the viability of cultured gingival keratinocytes decreased progressively with increasing concentrations of chlorhexidine, whereby the decrease of viability, similar to that in our study, was achieved at a concentration of chlorhexidine of 0.04 mg / ml. In our study, the same antiseptic showed this level of cytotoxicity (65%) after one minute but the concentration of the active substance

was much higher. Balloni et al. [13] in their experiment also used oral gingival keratinocytes and found out to ten times lower concentration of chlorhexidine after thirty seconds of exposure than we used in our experiment, the cytotoxic effect of chlorhexidine on keratinocytes in culture was such that it caused the decrease of viability of the cells to 61%.

Conclusion

Although chlorhexidine is considered the "gold standard" of oral antiseptics, in our study Octenisept* was showed as milder when it came to negative effect on keratinocytes of oral mucosa and thus can be considered the solution with better antiseptic properties. This can only be explained with different mechanisms of action between these two active substances. Chlorhexidine, due to its composition is an aggressive chemical, while Octenisept* is an antibiotic. We expected that antibiotic should have milder effect on living cells which is true when cytotoxic effect of chlorhexidine and Octenisept were compared but in our study Ozosept*, which is also a mixture of aggressive chemicals, was proven as the least aggressive towards isolated keratinocytes. This is a bit unexpected because Ozosept* is also a mixture of aggressive chemicals like chlorhexidine, not antibiotic like Octenisept*.

The presence of "transitional forms" of cells in our study of cytotoxic effect of Octenisept* and Ozosept* is another clear indicator of a lower degree of cytotoxicity of the active substance, especially as these forms have not been verified in the experiment with chlorhexidine.

Based on the above mentioned, we can conclude that Ozosept* and Octenisept* have less cytotoxic effect on isolated buccal keratinocytes while still having a proven anti-microbial effect and may represent good alternative to chlorhexidine. More comprehensive research on the fact mentioned is certainly needed.

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