

The Influence of Some Synthesis Compounds on Healthy and Tumoral Oral Cell Lines: PGK, HGF and SCC4

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This present study aimed to assess the influence of different chemical compounds which are commonly found in the composition of products used for oral hygiene on primary gingival keratinocyte, human gingival fibroblast and tongue squamous cell carcinoma behaviour. Chlorhexidine (Chx), aspartame (Asp), xylitol (Xyl) and sodium bicarbonate (NaB) were evaluated in an in vitro research regarding cells morphology and cytotoxicity. Primary gingival keratinocytes (PGK) and fibroblasts (HGF) treated with different concentration of the tested compounds did not show significant changes, nor related to morphology nor related to viability at the lowest concentrations used ($< 25\mu\text{M}$). In contrast, the tumor cells were affected by the test compounds, especially xylitol and sodium bicarbonate, both in terms of morphology and cytotoxicity, at concentrations over $5\mu\text{M}$.

Keywords: chlorhexidine, aspartame, xylitol, sodium bicarbonate, oral cells

Oral care products (for teeth and mouth care) are available as toothpaste, mouthwash, tooth whitening products, denture care products etc. Before placing on the market, they pass through standard safety assessments and are approved by regulatory bodies. However, in various situations, side effects have been reported. Specialty studies that treat biocompatibility and adverse reactions due to these types of products are in minority compared to those that treat their efficacy. The main reasons are: (i) the passive oral exposure of tissues to these products due to the short contact period between them and the preferential sponsorship of research which aim to evaluate the positive regards of oral care products in terms of efficacy and clinical performance compared to research which aim to assess adverse effects of these products [1].

Chlorhexidine is a bisbiguanide base, used as antiseptic with an important antimicrobial activity which is utilized in different forms (digluconate, hydrochloride, and acetate) being present in mouth rinse - 0.2%, gel - 1%, spray - 0.2%, toothpaste - 1%, root canal irrigant - 2%, varnishes, and periodontal chips (as antimicrobial agent) [1]. Even if it is the gold standard in his area exerts some oral side effects like: brown staining of teeth, mucosa, and restorations [2], taste disturbance [3], oral mucosal erosion, parotid swelling, and enhanced supragingival calculus formation [4].

Aspartame (E951), was discovered in 1965 and is synthesized through the combination of amino acids, namely L-phenylalanine, L-aspartic acid, and connected through methyl ester bonds being considered a source of phenylalanine (which must be considered for people with phenylketonuria) [5]. In literature, are data which describe the toxicity and hepatocellular alteration in long-term exposure to it [6]. Currently is investigated for its safety, even if was generally recognized as safe. Some pathologies are linked with his nephrotoxicity, hepatotoxicity, and damage to nerves, cancer, and even type 2 diabetes [7-10].

Xylitol (E967), a five-Cpolyol, synthesized by hydrogenation of xylose (for the first time in 1891) presents

around 95% sweetness of sucrose being the sweetest compound from its class, contributing with 2.4 kcal/g. Can be also obtained by different extraction methods from birch and other woods, almond husks, corncobs, straw and paper production surplus. Can be found in many fruits and vegetables [1,10].

Sodium bicarbonate exerts an antibacterial effect because presents cleansing action by loosening debris and dissolving mucus. Due to the increase values of the pH in the oral cavity after administration of the compound, can be prevented the overgrowth of acidophilic bacteria. Some studies pointed out that mouthwash with NaHCO_3 1% causes less irritation to the oral mucosa in a group of sensitive cancer patients as compared with other similar products [1].

The present study was proposed to assess the activity on normal and tumoral oral cell lines of four different chemical compounds found in oral care products: chlorhexidine (Chx), aspartame (Asp), xylitol (Xyl) and sodium bicarbonate (NaB).

Experimental part

Materials and methods

Chemicals and reagents

All chemical reagents and standard substances used were of analytical reagent grade and obtained from Sigma-Aldrich and Merck (Germany). The chemical compounds stock solutions used were initially dissolved in complete medium, Dulbecco's Modified Eagles Medium (DMEM) at concentrations of 10 mM for chlorhexidine and sodium bicarbonate, and 1 mM for aspartame and xylitol, respectively. After preparation, the stock solutions were filtered through a 0.22mm filter and diluted with medium in order to obtain the final working concentrations (adjusted to pH 7.4) used within the tests.

Cells culture

The cell lines utilized in the current experiments were primary gingival keratinocytes and fibroblasts (normal/

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healthy cells - PGK and HGF) and tongue carcinoma (tumor squamous cells - SCC4) purchased from American Type cell Collection, commercial forms - frozen vials. The specific medium for cells culture and kit, like: Dermal Cell Basal Medium (DMEM), Fibroblast Basal Medium (FBM) and Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DME:F12), Keratinocyte Growth Kit and MTT were supplied from Sigma Aldrich and ATCC. Other characteristic reagents among which fetal bovine serum (FBS), phosphate saline buffer (PSB), trypsin, ethylenediaminetetraacetic acid (EDTA), penicillin and streptomycin mixture, Trypan Blue, hydrocortisone were purchased from Sigma Aldrich, Merck and ATCC.

The cells exposed to various concentrations of Chx, Asp, Xyl, NaB (1, 5, 10, 25 and 50 μ M) over three time periods (24 hours) were examined under a phase-contrast microscope for any changes in their attachment to the culture flask, size, shape, integrity of their cell characteristics.

The MTT cell viability assay was used and the protocol was adapted from the literature [11]. In brief, the cells were seeded in 96-well cell culture plates and exposed to the test compounds as mentioned above. After incubation period (24 hours), the MTT solution was added to each well and the culture plates were incubated at 37°C for 4 hours and after removal of the liquids from the wells DMSO was added in order to dissolve the formazin crystals and the absorbance was read at 540nm with a reference wavelength of 690nm. Results were expressed as % cell viability.

Statistical analysis

The statistical and software programs applied in the present study were GraphPad Prism 7. Data were processed using One-way Anova followed by Tukey's post-tests, in order to establish the statistical difference groups; *, **, *** and **** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively.

Results and discussions

Cells morphology.

The cells were observed to have an intact morphology and a similar size and shape as the untreated controls up to a concentration of chemical compounds of approximately 25 μ M. Cells presented clear morphological changes when observed under the phase-contrast microscope after exposure to test compounds in excess of 50 μ M, after 24 h post-stimulation. They lose their well-defined form as can be observed in the figures 1-3.

Cells viability.

The effect on viability of PGK, HGF and SCC4 cells treated with different concentrations of the four chemical compounds chlorhexidine, aspartame, xylitol and sodium bicarbonate, over 24 h was assessed by using MTT method. All the compounds caused a decrease in cell viability of the tumor cells in a concentration and time dependent manner as compared to controls, however to varying degrees. A slightly higher cell viability, as compared to untreated cells, was observed for healthy all cells- PGK

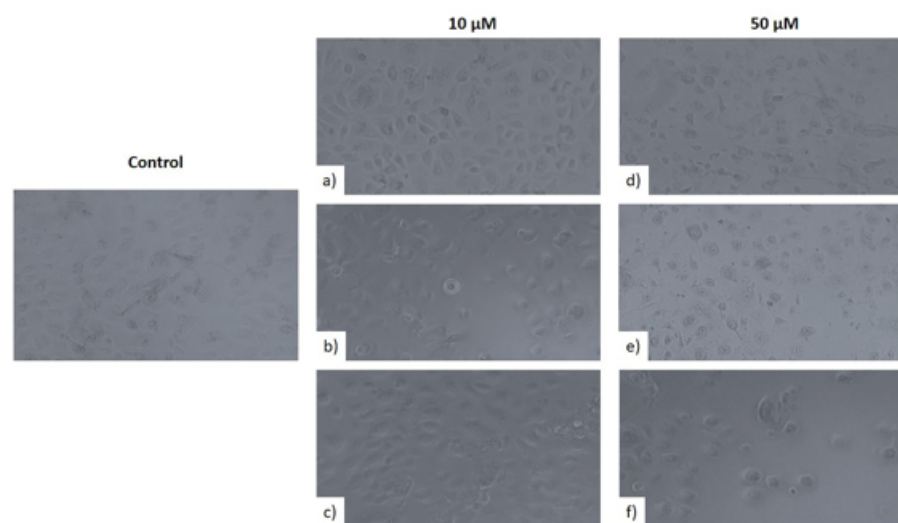


Fig. 1. Morphology of gingival keratinocytes cells at 24h post-stimulation with test compounds: a) and d) chlorhexidine; b) and e) aspartame; c) and f) xylitol

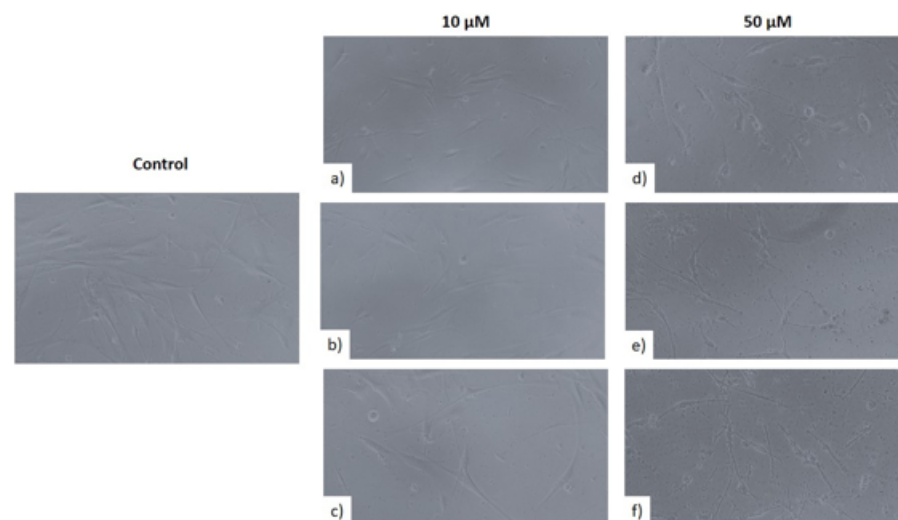


Fig. 2. Morphology of gingival fibroblasts cells at 24h post-stimulation with test compounds: a) and d) chlorhexidine; b) and e) aspartame; c) and f) xylitol

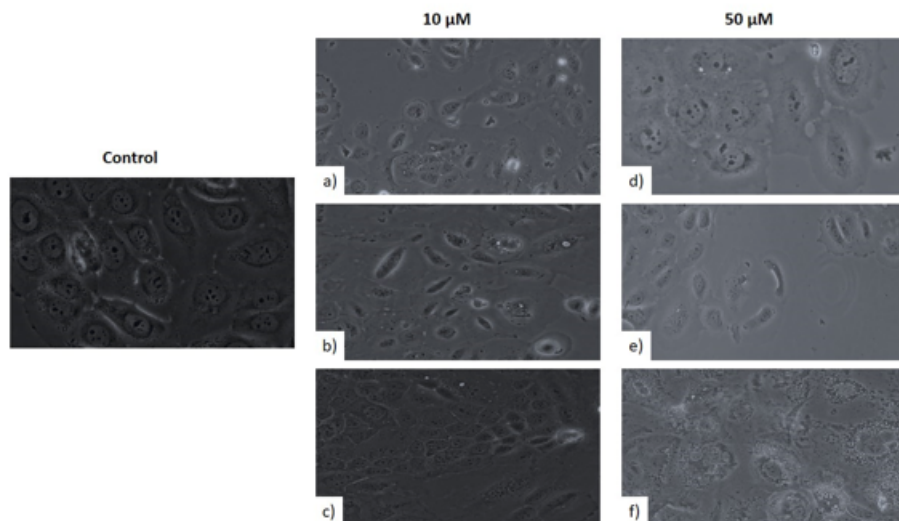


Fig.3. Morphology of tongue squamous carcinoma cells at 24h post-stimulation with test compounds: a) and d) chlorhexidine; b) and e) aspartame; c) and f) sodium bicarbonate

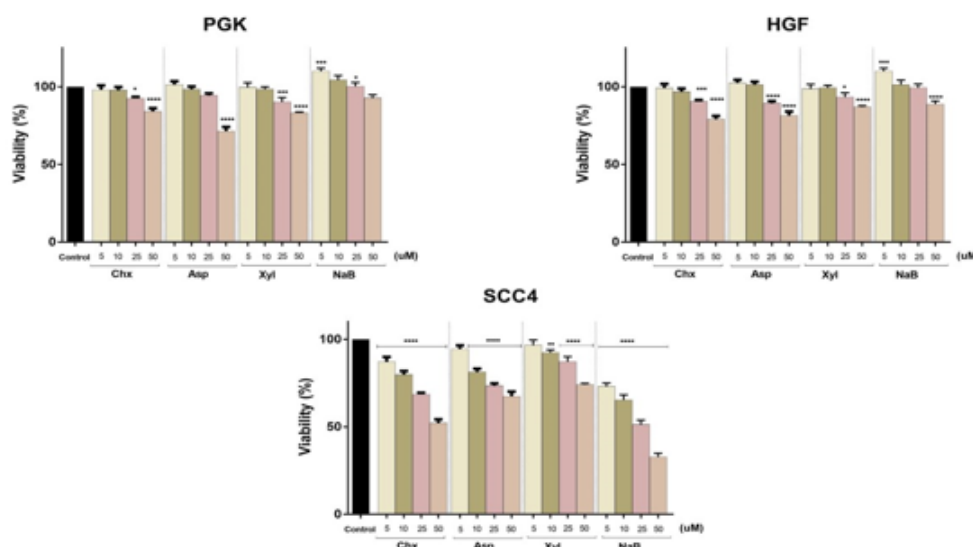


Fig. 4. Viability percentage of primary gingival keratinocytes (PGK) and fibroblasts (HGF) and squamous carcinoma (SCC4) at 24h post-stimulation with test compounds (5, 10, 25 and 50 μ M)

and HGF-when treated with low concentrations of sodium bicarbonate (1-10 μ M), however exposure to chemical compounds applied in higher concentrations caused decreased cell viability in a dose-dependent manner. In case of PGK after post-stimulation with the compounds at 50 μ M the viability was as follows: ~ 84% for Chx, ~ 71% for Asp, ~ 83% for Xyl and ~ 92% for NaB while regarding HGF the percentage were: ~ 79% for Chx, ~ 81% for Asp, ~ 87% for Xyl and ~ 88% for NaB

After 24 h of incubation, no statistically significant differences could be detected between the following compounds, chlorhexidine, aspartame and xylitol at 5 and 10 μ M concentrations. The test compounds concentrations starting at 10 μ M caused a decrease in cell viability in a dose-dependent manner for the SCC4 cells, and as can be seen in the figure 4, this cell line seemed to be more sensitive to all the compounds except xylitol compared to the healthy cell lines. For the SCC4 cell line the greatest decrease in cell viability was observed with chlorhexidine (~ 68% at 25 μ M and ~ 52% at 50 μ M) and sodium bicarbonate (~ 51% at 25 μ M and ~ 33% at 50 μ M).

Aspartame at low doses can be considered a potential angiogenic compound that can produce regenerative cytokine production. It enhances the specific markers and receptors and finally can be formed new blood vessels, which develop proper conditions that *in vivo* may result in the promotion of specific pathologies such as: diabetic retinopathies, rheumatoid arthritis and tumor cell invasion and spreading of metastases [12].

Some authors, like Park and collaborators stated that xylitol inhibited the proliferation of cancer cells such as A549, Caki, NCI-H23, HCT-15, HL-60, K562, and SK MEL-2 cells, but had less effect on the cell viability of HGF cells and also induces cell morphological changes and autophagy in lung cancer cells [13].

All these preliminary studies may have clinical relevance only after correlating with other *in vivo* detailed studies due to the fact that the utilized cells do not have the salivary pellicle layer, and cannot reproduce the immunologic issues *in vivo* or the protective barriers at the tissue level [14].

Conclusions

Chemical compounds, like disinfectants and artificial sweeteners are currently classified as safe for consumption in various products by many international regulatory bodies, while some of them are still classified to be used 'with caution' in some countries. The results obtained in this study offer important data regarding the influence of Chx, Asp, Xyl and NaB on cell lines behaviour but they must be extrapolated to the *in vivo* conditions. The present study serves to provide further information on the possible effect these agents have on mammalian cells, however further studies will have to be performed to clarify what exactly these effects might be.

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