Effects of Na-DNA mouthwash solutions on oral soft tissues - a bioreactor-based Reconstituted Human Oral Epithelium model

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Conflict of interest statement

The Authors report no conflict of interest.

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ABSTRACT: Purpose: This study aimed to investigate whether the addition of Na-DNA to chlorhexidine (CHX)-containing mouthwash influences morphology and viability of a Reconstituted Human Oral Epithelium (ROE), and protects ROE against oxidative stress. **Methods:** Multi-layered 0.5 cm^2 ROE specimens were positioned inside a continuous-flow bioreactor and grown air-lifted for 24 h. They were treated with phosphate-buffered saline (PBS) (n=16) or 1 vol% H_2O_2 for 1 min (n=16), then, they were treated for 5 (n=8) or 30 min (n=8) with experimental mouthwash solutions containing: 0.2 % CHX, 0.2 % CHX + 0.2 wt% Na-DNA, 0.2 wt% Na-DNA, PBS. After 60 min washout specimens were subjected to tetrazolium-based viability assay (MTT) Confocal Laser-Scanning Microscopy (CLSM), and histological evaluation using optical microscopy and Transmission Electron Microscopy (TEM). **Results:** ROE treated with Na-DNA for 30 min revealed significantly higher viability than PBS, and CHX + Na-DNA showed higher viability after 30 min treatment than after 5 min, suggesting a significant protective activity of Na-DNA. Moreover, the protective effect of Na-DNA on cell viability was higher after the induction of oxidative stress. After treatment with CHX, CLSM revealed cell suffering, leading to cell death in the outer layer. On the contrary, specimens treated with Na-DNA showed a much lower amount of dead cells compared to PBS, both in the absence or presence of oxidative stress. Histological examination showed that the protective action of Na-DNA formulations reached more in-depth into the epithelium exposed to oxidative stress, due to intercellular spaces opening in the outer epithelium layers, giving way to Na-DNA to the inner parts of the epithelium. It can be concluded that Na-DNA had a topical protective activity when applied for 30 min unless the epithelium barrier is damaged, allowing it to act more in-depth.

CLINICAL SIGNIFICANCE: Na-DNA showed a clear and protective action against cellular degeneration due to oxidative stress and, partly, to the exposure to CHX. Its addition to chlorhexidine mouthwash or gels could be clinically helpful in contrasting the detrimental activity of CHX on oral tissues, and in the preservation of cell viability, control of inflammation and wound healing.

Introduction

Maintaining good oral hygiene is a fundamental issue in everyday life and especially during dental treatments. Even though formulations and recipes for the benefit of oral and dental health existed for at least 6000 years, the term "oral hygiene products" is relatively recent [1]. During all this period, most toothpowders, toothpastes, and mouthwashes appeared to have been formulated for cosmetic reasons predominantly, including tooth cleaning/whitening and breathe freshening, rather than for medical reasons such as controlling dental and periodontal diseases, and soft tissue healing [2]. Considering the easiness of use of mouthwashes and toothpastes, they are the ideal vehicles for bioactive/biocidal agents [3].

Due to its antibacterial activity, chlorhexidine (CHX) is the primary agent able to prevent plaque formation [4 5], representing the most investigated and prescribed product. Its use is mainly intended for the treatment of periodontal diseases and maintenance therapy as well as for immediate postoperative management [6 7]. *In vitro* studies proved that low CHX concentrations could damage bacterial cell membranes, while high concentrations cause the precipitation and coagulation of cytoplasmic proteins [5]. The efficacy of CHX against bacteria, mycetes, and viruses is widely known [4] as well as its ability to firmly adsorb to tooth structures and oral epithelium (substantivity) [8]. Side-effects of CHX, in the most commonly used concentrations, are considered to be very few and include inflammatory reactions, dysgeusia, and tooth pigmentations [6].

However, its effects on tissues, particularly during the healing processes, have been questioned by a few studies. The use of CHX has been reported to cause tissue necrosis and inhibition of regeneration [9 10 11 12 13]. Moreover, different studies demonstrated that CHX was able to induce primary DNA damage in rat leukocytes and oral mucosal cells, providing evidence for genotoxicity [14 15]. Considering these drawbacks, recent research is increasingly focusing on methods that can prevent or decrease the cytotoxic effects of that substance, while maintaining its activity. In this context, nucleotides, and nucleosides are assuming an essential role due to their protective effect against cell damage [16].

Sodium DNA (Na-DNA) is a relatively new, biologically active, functional compound. It is constituted by native deoxyribonucleic acid extracted from the gonadic tissue of male sturgeons, which has been purified, depolymerized, and neutralized with sodium ions. The Na-DNA molecule can enter the cells through pinocytosis [17]. Once inside, the nucleotides act as a structural base for the biosynthesis of nucleic acid and low molecular weight co-factors. This compound has been found to increase proliferation and activity of different cell types by acting in synergy with several growth factors (i.e., epidermal growth factor, EGF, platelet-derived growth factor, PdGF, and fibroblast growth factor, FGF), modulating cytokines and growth factor production, and influencing immunological response [18 19 20 21]. The result of such activity is a repairing action related to the upregulation of cytokines and growth factors in cells under stress or metabolic alteration [20 21 22 23].

Nevertheless, the high variability of the interindividual response (i.e. upregulation/downregulation in cell pathways) to active compounds makes the testing of such agents challenging and time-consuming when considering *in vivo* studies, even when safetiness of such agents has been demonstrated. Reconstituted Oral Epithelium is a practical and recent tool to study the effect of different active compounds on soft tissues, mimicking the behavior of human mucosa and allowing for testing of active compounds in a standardized way [24].

The present study aimed to investigate whether the presence of Na-DNA in the formulation of CHX-containing mouthwashes could reduce cytotoxic effects of CHX on a human reconstituted oral epithelium, and to protect cells against oxidative stress.

Materials and methods

Reagents

Reagents, culture media, and disposables used in this study were obtained from Merck (E.Merck AG, Darmstadt, Germany). ROE specimens (0.5 cm², SkinEthic HOE™/Human Oral Epithelium) were obtained from EPISKIN (EPISKIN, Lyon Cedex 7, France). Na-DNA (Kalinat® AW) was obtained from KALICHEM (Kalichem, Brescia, Italy). The following mouthwash solutions, not containing any preservatives, were tested:

A. 0.2% CHX-containing mouthwash (positive control)
B. 0.2% CHX-containing mouthwash + 0.2 wt% Na-DNA (test)
C. 0.2 wt% Na-DNA (test)
D. Phosphate-buffered saline (PBS, negative control)

Mouthwash solutions were inserted into identical flasks that were coded with an alphabetical letter from A to D, ensuring that experimenters were blinded regarding the composition or the effect of the solutions.

Reconstituted Human Oral Epithelium (ROE)

A total of 32 specimens of ROE were used for the study. Specimen were shipped in 24-well plates containing agarose-nutrient transport medium. Upon arrival in the laboratory, the bags containing the ROE specimens were opened under sterile airflow hood. The specimens were extracted from the transport plates, and the agarose was removed. Then specimens were placed in 6-well plates with nutrient medium (RPMI 1640 medium, supplemented with 20.0 % fetal bovine serum, 1.0 % L-glutamine, and 1.0 % penicillin/streptomycin). Before testing, the culture plates were incubated overnight at 37 °C, in a 5 % CO_2 atmosphere and saturated humidity.

Bioreactor

A Modified Drip-flow Bioreactor (MDFR) was used for this study (Fig. 1). The device is a modification of a commercially available Drip Flow Reactor (DFR 110; BioSurface Technologies, Bozeman, MT, USA). The modified design allowed the placement of customized trays on the bottom of the flow cells and the immersion of ROE specimens into the surrounding flowing medium [25 26] . This allowed the use of nutrient medium at a continuous flow rate.

All tubing and specimen-containing trays of MDFR were sterilized before the beginning of the experiment using a chemiclave with hydrogen peroxide-based sterilization system (Sterrad; ASP, Irvine, CA, USA). By limiting the maximum temperature to 45°C, heat-related damage to the whole system is avoided. The MDFR was then assembled inside a sterile hood.

The specimens were cut out from their carrier using sterile scalpel and tweezers and placed into eight polytetrafluoroethylene (PTFE) trays containing four holes each, which fixed them and exposed their surfaces to the flow medium. All trays were fixed on the bottom of each of the flow

chambers of two MDFRs running in parallel and immediately inoculated with fresh nutrient medium. The MDFR was transferred into an incubator operating at 37 °C, 5 % of CO_2, and 100% relative humidity atmosphere. Then a multichannel, computer-controlled peristaltic pump (RP-1; Rainin, Emeryville, CA, USA) was turned on and used to provide a constant flow of nutrient medium through the flow cells. The flow rate was set to 9.6 ml/h.

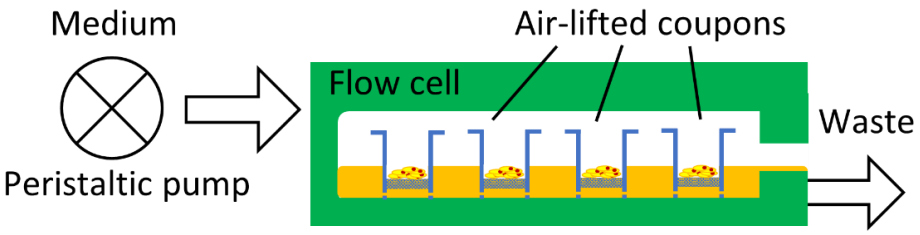

Fig. 1. Functioning diagram of the coupon-bearing bioreactor. The peristaltic pump provides a constant flow of supplemented medium to the flow cells for the specified amount of time. It was possible to treat the two specimens close to the output of the bioreactor for a longer time (30 min) than the other two (5 min) by inclining the bioreactor clockwise for 25 min.

Test procedures

After an additional 24h, the pump was stopped, and four flow cells were treated with the tested mouthwash solutions (A, B, C, or D), one for each flow-cell (10 ml). In each flow cell, two specimens were treated for 5 min and the other two for 30 min by inclining the bioreactor for 25 min, so that the solution completely covered the bottom two specimens, then setting it again in a horizontal position for the remaining 5 min to cover all four specimens. The remaining four flow cells were first treated with a 1 vol% H_2O_2 solution for 1 min to induce high oxidative stress and cell damage, then specimens were thoroughly rinsed with sterile PBS for 1 min, and the flow cells were treated with the tested mouthwash solutions as previously specified. Again, in each flow cell, two specimens were treated with the mouthwash solutions for 5 min, and the other two for 30 min (Fig. 2). After that, the pump was turned on again for washout of the mouthwash solutions for 60 min, and then all ROE specimens were extracted from the flow-cells, immediately cut into four equal parts using sterile scalpel and tweezers and processed as follows.

Specimens evaluation

ROE specimens from each mouthwash treatment and evaluation were quantitatively evaluated using MTT viability assay (n=4). Morphological analysis of the specimens was performed using Confocal Laser-Scanning Microscopy (CLSM) imaging (n=2), and histological evaluation (n=2) using optical microscopy and Transmission Electron Microscopy (TEM) imaging (Fig. 2).

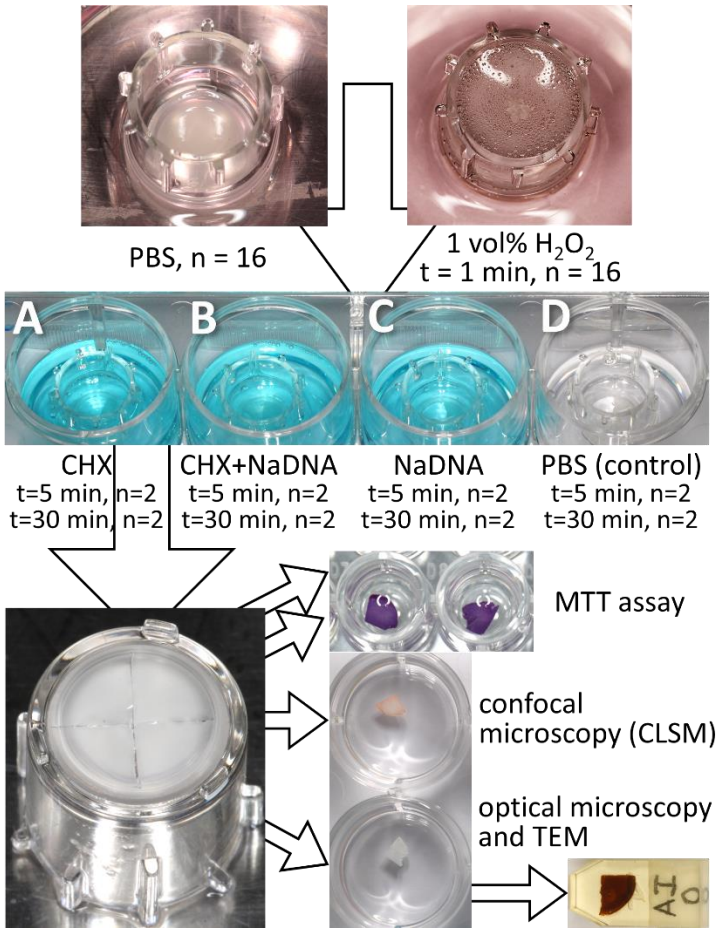

Fig. 2. Diagram representing the test procedures and specimens evaluation. Half of the specimens were subjected to high oxidative stress; then, they were treated with one of the tested solutions for either 5 min or 30 min. Then, each coupon was cut into four equal parts using a scalpel blade and sterile tweezers and subjected to viable biomass quantitative assessment (MTT assay), morphological and viability assessment (CLSM), and histological macro- and ultrastructural analysis using optical microscopy and TEM.

MTT assay

Cell survival was evaluated via MTT viability assay [26 27]. The assay was performed as follows: two starter stock solutions were prepared by dissolving 5.0 mg/ml 3-(4,5)-dimethylthiazol-2-yl-2,5-difenyltetrazolium bromide (MTT) in sterile PBS, and 0.3 mg/ml of N-methylfenazinium methyl sulfate (PMS) in sterile PBS. The solutions were stored at 2 °C in lightproof vials until the day of the experiment when a fresh measurement solution (FMS) was made by mixing on a 1:1:8 ratio, respectively, MTT stock solution, PMS stock solution, and sterile PBS. A lysing solution (LS) was prepared by dissolving 10 % v/v of sodium dodecyl sulfate and 50 % v/v of dimethylformamide in distilled water. ROE specimens subjected to MTT assay were placed inside the wells of a sterile, flat-bottomed 24-well plate. After that, 1 ml of FMS was pipetted into each well, and the plates were incubated at 37 °C in lightproof conditions for 1 h. During incubation, electron transport across the cell membrane and, to a lesser extent, cellular redox systems converted the yellow MTT salt to insoluble purple formazan. The conversion was facilitated by the intermediate electron

acceptor (PMS). The unreacted FMS was then gently removed from the wells by aspiration. The formazan crystals were dissolved by adding 1 ml of LS into each well, followed by additional incubation in lightproof conditions at room temperature for 1 h. A total of 100 µl of the suspension was then removed from each well, and optical density (at 550 nm) was measured with a spectrophotometer (Genesys 10-S, Thermo Spectronic, Rochester, NY, USA).

CLSM observations

CLSM imaging using Live/Dead staining was performed as described [28]. Briefly, the ROE specimens subjected to CLSM observations were stained using the LIVE/DEAD® Viability Kit for microscopy (Invitrogen Ltd., Paisley, UK). The fluorescence from live stained cells was observed using a CLSM (Eclipse Ti2 inverted CLSM, Nikon, Tokyo, Japan). Four randomly selected image stack sections were recorded for each ROE specimen. Confocal images were obtained using a dry Plan Apochromat 20x (NA 0.75) objective and digitalized using Nikon proprietary software (NIS), at a resolution of 1024 × 1024 pixels, with a zoom factor of 1.0. For each image stack section, 3D-rendering reconstructions were obtained using Drishti 3D software [29].

Histological evaluation

ROE specimens undergoing histological analysis were fixed overnight in freshly prepared Karnovsky solution (2.0 vol% paraformaldehyde, 2.0 vol% glutaraldehyde in 0.1 M sodium cacodylate buffer).
After rinsing in the cacodylate buffer, specimens were postfixed with 2.0 wt% OsO_4 and stained *en-block* with 2.0 wt% uranyl acetate. They were then dehydrated in graded acetone solutions and embedded in Epon-Araldite resin (EMS, Hatfield, PA, USA). Semi-thin (0.5 µm) transverse sections of the entire specimen profiles from the different experimental groups were obtained using an ultramicrotome (Leica Supernova, Reichert Ultracut, Leica Microsystems, Wetzlar, Germany) and stained using toluidine blue. Then, they were examined using a digitalized light microscope with a 63x objective (Zeiss Axiophot microscope, Oberkochen, Germany) and by TEM (Zeiss EM10 microscope, Oberkochen, Germany) at a final magnification of 1500x.

Statistical analysis

MTT assay dataset was preliminarily checked for normality of distribution (Shapiro-Wilk's test) and homoscedasticity (Levène's test). Since data were not normally distributed even after log-transformation, a nonparametric analysis was performed using Wilcoxon's test ($p<0.05$).

Results

MTT assay

The results of the MTT assay are displayed in Figure 3. After 5 min treatment with the mouthwash solutions, a significant decrease in viability was observed in specimens treated with solution C (0.2 wt% Na-DNA) confronted to solution B (0.2% CHX-containing mouthwash + 0.2 wt% Na-DNA). No significant change in the viability of the specimens was identified in comparison to the negative control (PBS). Treating ROE specimens with the mouthwash solutions for 30 min resulted in a significant decrease in viability by solution C compared to the CHX-containing solutions. The

incubation with 1 vol% H_2O_2 unsurprisingly lead to a general decrease in viability. Specimens treated with mouthwash solutions A for 5 min after the oxidative stress showed, however, significantly higher viability compared to solution B.

After 30 min of treatment with the mouthwash solutions following oxidative stress, significantly higher viability of specimens treated with both solutions A and B compared to solutions C and D could be demonstrated. Furthermore, solution C elicited significantly higher viability than solution D.

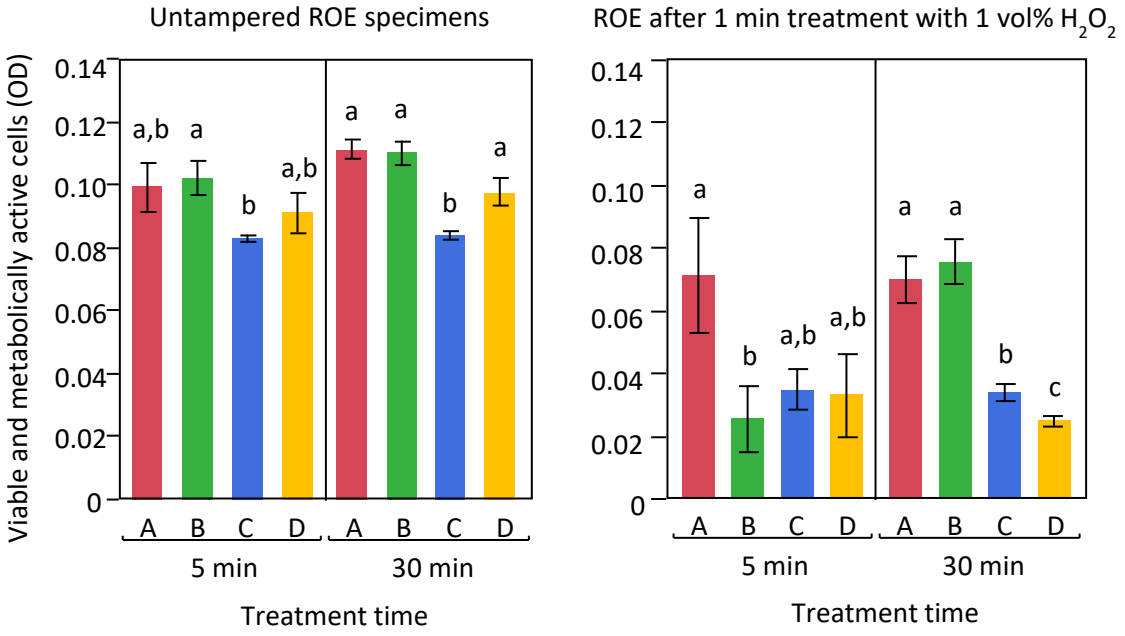

Fig. 3. Viable and metabolically active ROE cells. Optical density units (means ± 1 standard error) are displayed. The results are displayed after treatment with: 0.2% CHX-containing mouthwash (solution A, positive control); 0.2% CHX-containing mouthwash + 0.2 wt% Na-DNA (solution B); 0.2 wt% Na-DNA (solution C) and PBS (solution D, negative control). For each treatment time, different superscript letters indicate statistically significant differences between groups (Wilcoxon test, $p<0.05$).

CLSM observations

Confocal microscopy reconstructions obtained after 5 min treatment with the mouthwash solutions did not show differences between groups (data not shown). Reconstructions of specimens after 30 min incubations are shown in Figure 4 (untampered specimens) and Figure 5 (specimens after 1 min H_2O_2 treatment).

In all specimens, due to the tight junctions between cells, dye penetration was limited to the first and most external layers. Specimens treated with mouthwash solution A and, to a lesser extent, B, showed good preservation of the cell structure even in specimens exposed to hydrogen peroxide. However, limits between cells were clearly identifiable, meaning that junctions between cells were loosely tightened. Negative control specimens (D) showed the presence of dead cells on the surface, and after hydrogen peroxide treatment, the most external layer was almost completely composed of

dead cells. Specimens treated with Na-DNA (solution C) showed a much lower amount of dead cells compared to the negative control both in the absence or presence of hydrogen peroxide treatment.

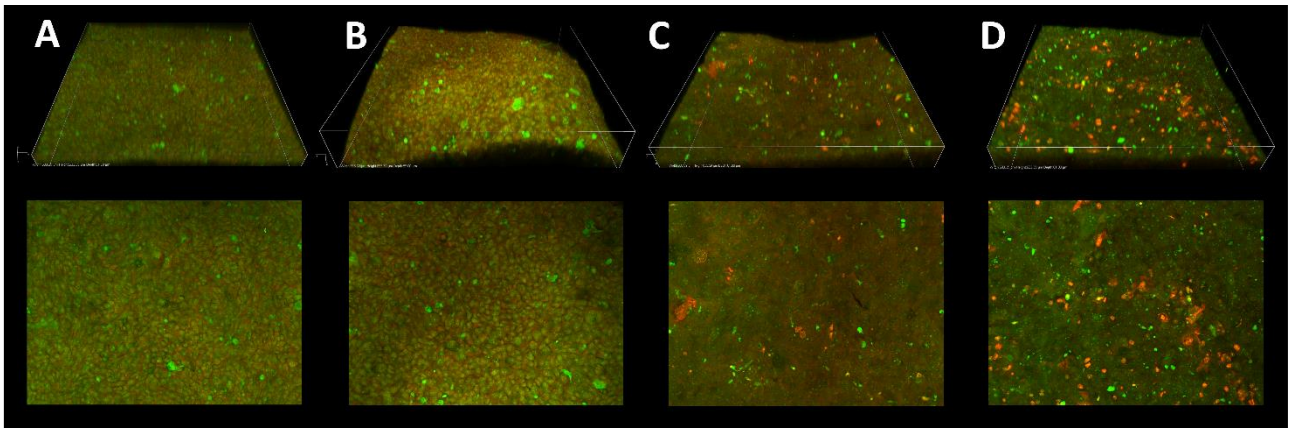

Fig. 4 shows the 3D reconstructions of CLSM images (upper panels, 1270 x 1270 μm) and Maximum Intensity Projections (lower panels) of ROE specimens after 30 min treatment with the corresponding mouthwash solution. Live cells fluoresce bright green, whereas dead cells with compromised membranes fluoresce red. Each picture letter indicates the corresponding mouthwash treatment. A: 0.2% CHX-containing mouthwash (positive control); B: 0.2% CHX-containing mouthwash + 0.2 wt% Na-DNA; C: 0.2 wt% Na-DNA, and D: PBS (negative control). Initial enlargement of intercellular spaces, meaning cell suffering, can be seen in CHX-treated groups. A lower amount of dead cells in the superficial layer can be found after Na-DNA treatment compared to the control.

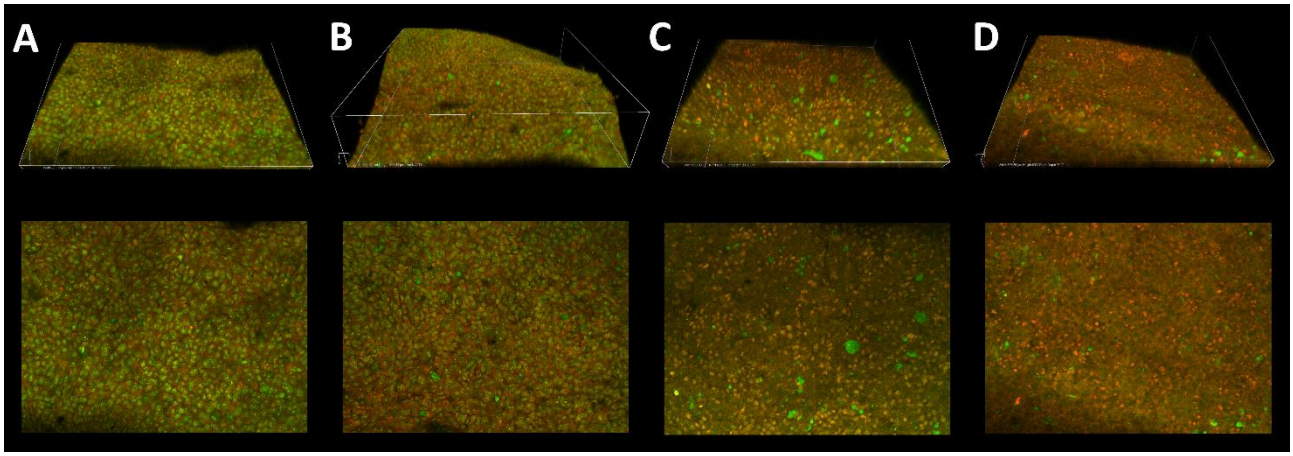

Fig. 5. CLSM 3D reconstructions (upper panels, 1270 x 1270 μm) and Maximum Intensity Projections (lower panels) of ROE specimens after 1 min treatment with H_2O_2, then 30 min treatment with the mouthwash solutions. Live cells fluoresce bright green, whereas dead cells with compromised membranes fluoresce red. Each picture letter indicates the corresponding mouthwash treatment. A: 0.2% CHX-containing mouthwash (positive control); B: 0.2% CHX-containing

mouthwash + 0.2 wt% Na-DNA; C: 0.2 wt% Na-DNA and D: PBS (negative control). In all fields, cell junctions were compromised due to H_2O_2 treatment, which allowed dyes to penetrate more deeply into the tissue. Also, the red signal was generally more intense, especially in D, meaning that a lower amount of dead cells is present after Na-DNA treatment when compared to the control.

Histological evaluation

ROE tissue semi-thin sections obtained after 5 min treatment with the mouthwash solutions did not show differences between groups (data not shown). Sections of the specimens after 30 min treatment are shown in Figure 6 (untampered specimens) and Figure 7 (specimens after 1 min H_2O_2 treatment).

Considering the untampered specimens, the negative control (D) showed the complete preservation of tissue structures. Specimens treated with mouthwash solutions A and, to a lesser extent, B, showed both outermost and basal cell layers presenting damage to the cell structure, such as cytoplasmic vacuolizations, altered nucleus, and initial enlargement of the intercellular spaces. These alterations can be due to CHX activity. Na-DNA addition to the CHX mouthwash showed less intense cell structure alterations compared to CHX-only mouthwash. Specimens treated with mouthwash solution C showed the same alterations, yet limited to the first cell layer. Hydrogen peroxide treatment displayed, as expected, extensive damage to ROE cells, such as marked vacuolization, degenerated nucleus, and enlargement of the intercellular spaces. These alterations were most noticeable in specimens treated with the negative control (D) and with mouthwash solution A (CHX only). In specimens treated with mouthwash solution B, some cells not showing degeneration signs can be found in the innermost layers, where they were more protected from the reactive oxygen species generated by hydrogen peroxide, and where Na-DNA helped in minimizing cell damage. Specimens treated with mouthwash C (0.2 wt% Na-DNA only) showed the least signs of cellular degeneration.

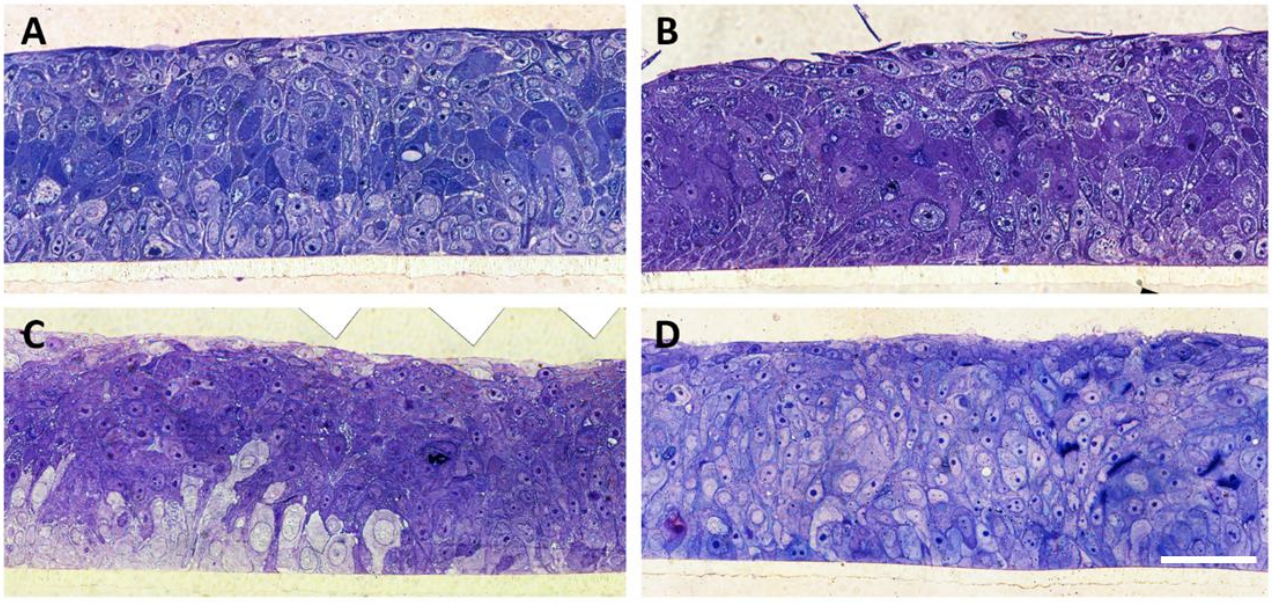

Fig. 6. Semi-thin sections of ROE specimens after 30 min treatment with the mouthwash solutions. The outer layer of the epithelium is oriented upwards (Scale bar: 50 µm). Each picture letter indicates the corresponding mouthwash treatment. A: 0.2% CHX-containing mouthwash (positive control); B: 0.2% CHX-containing mouthwash + 0.2 wt% Na-DNA; C: 0.2 wt% Na-DNA and D: PBS (negative control). Higher magnification fields were acquired, then photo-stitching and contrast optimization were performed to obtain high-resolution fields spanning the whole thickness of the epithelium.

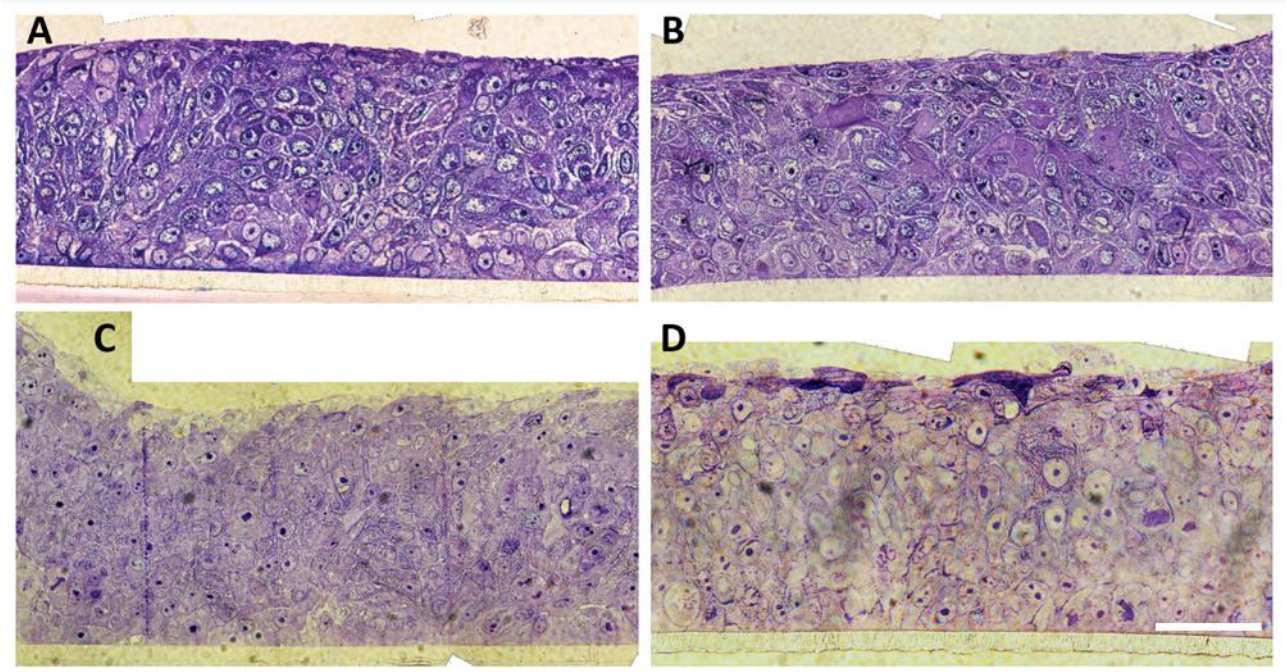

Fig. 7. Semi-thin sections of ROE specimens after 1 min treatment with H_2O_2, then 30 min treatment with the mouthwash solutions. The outer layer of the epithelium is oriented upwards (Scale bar: 50 µm). Each picture letter indicates the corresponding mouthwash treatment. A: 0.2% CHX-containing mouthwash (positive control); B: 0.2% CHX-containing mouthwash + 0.2 wt% Na-DNA; C: 0.2 wt% Na-DNA and D: PBS (negative control). Higher magnification fields were acquired, then photo-stitching and contrast optimization were performed to obtain high-resolution fields spanning the whole thickness of the epithelium.

TEM analysis

Specimens were observed using TEM to better investigate the fine morphology of the ROE after hydrogen peroxide treatment followed by 30 min application of the tested solutions (Fig. 8). Hydrogen peroxide treatment displayed, as expected, extensive damage to ROE cells, such as marked vacuolization, degenerated nucleus, and enlargement of the intercellular spaces. These alterations were most noticeable in specimens treated with D and A. In B and, especially, in C, some cells not showing degeneration signs can be found in the innermost layers, where they were more protected from the reactive oxygen species generated by hydrogen peroxide.

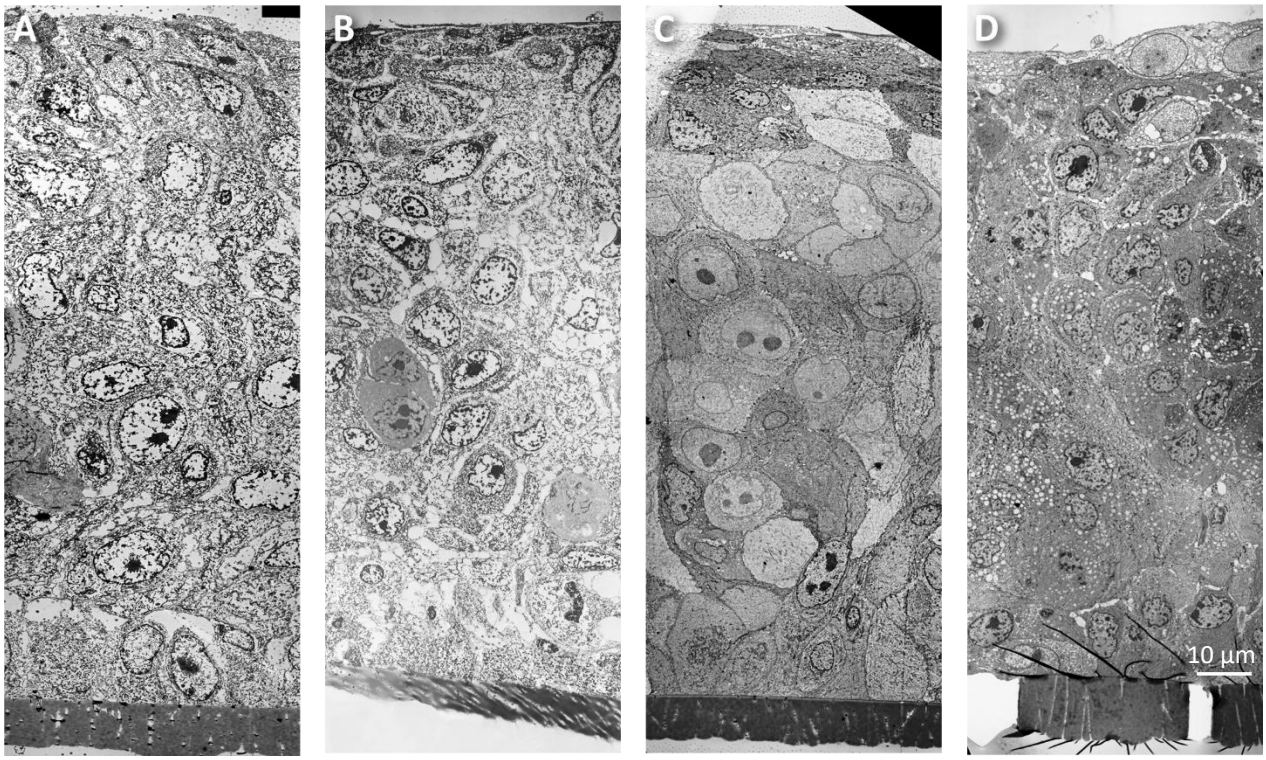

Fig. 8. TEM images of transverse ROE sections after 1 min treatment with H_2O_2, then 30 min treatment with the mouthwash solutions. The outer layer of the epithelium is oriented upwards. The different layers that characterize the human oral epithelium from the basal (bottom) to the keratinized surface layer (top) can be observed (Scale bar: 10 µm). Each picture letter indicates the corresponding mouthwash treatment. A: 0.2% CHX-containing mouthwash (positive control); B: 0.2% CHX-containing mouthwash + Na-DNA; C: Na-DNA and D: PBS (negative control). Higher magnification fields were acquired, then photo-stitching and contrast optimization were performed to obtain high-resolution fields spanning the whole thickness of the epithelium.

Discussion

Our research represents the first *in vitro* study evaluating the effects of Na-DNA treatment in combination with CHX-containing mouthwashes to protect cells against cytotoxicity and oxidative stress. We used a human ROE model developed inside a bioreactor to evaluate by MTT assay, light and electron microscopy, cell viability, and degeneration effect of CHX combined with or without Na-DNA. Furthermore, the same methodology was applied after challenging ROE specimens with oxidative stress. The efficacy of CHX-containing products is widely considered as the gold standard for the treatment of several biofilm-associated oral care diseases [4 5 6 7]. Nevertheless, our starting point was some literature evidence about the detrimental effects of CHX on oral tissues [14 15]. The present study, therefore, studied the possibility to minimize the adverse effects of CHX using a compound, Na-DNA, that demonstrated protective and repair cellular activity [16], but was not tested in the oral environment yet.

Our results showed that, after 30 min treatment, Na-DNA elicited significantly higher viability than the control, and CHX + Na-DNA showed higher viability after 30 min treatment than after 5 min, suggesting a significant protective activity of Na-DNA. Moreover, the protective effect of Na-DNA on cell viability was higher after the induction of oxidative stress. After treatment with CHX

mouthwashes, confocal microscopy showed the loss of cell junctions in the superficial cell layer, which was an indirect sign of cell suffering, leading to cell death in the outer layer. On the contrary, specimens treated with Na-DNA showed a much lower amount of dead cells compared to the negative control, both in the absence or presence of oxidative stress. The histological and ultrastructural evaluations showed that ROE treated with CHX and, to a lesser extent, CHX + Na-DNA, showed alterations of the cell structure, such as vacuolization, degenerated nucleus and initial enlargement of the intercellular spaces both in the outermost and basal cell layers. These alterations can be due to CHX activity. Specimens treated with Na-DNA showed the same alterations, yet limited to the first cell layers.

Surprisingly, we found significantly higher viability in specimens treated with mouthwashes containing CHX compared to Na-DNA or control after 30 min of treatment. These results suggest a direct influence of CHX on the response of the cell to the MTT test. The unexpected, higher amount of tetrazolium reduction shown by the CHX-containing mouthwash was most likely due to an effect of chlorhexidine itself. In fact, this was first reported by Lindhe et al. [30], who observed that chlorhexidine-treated mucosa caused a higher and substrate-independent formazan deposition compared to control mucosa, especially during the first hour after chlorhexidine application. Due to the activity of tetrazolium salts, they are sensitive to any compound with reducing capacities as well as to light exposure [27]. As a consequence, all tests determining cell viability with biochemical means have to be interpreted with caution to ascertain possible direct influences of an active principle on the reagents.

The detrimental activity of CHX that was evidenced in the present study is in agreement with the findings of several authors. This compound was associated with cytotoxic effects in gingival epithelial cells [31], in cells from the periodontal ligament [32], in keratinocytes [33 34], in macrophages [35], in osteoblasts and in osteoclasts [12 13]. In particular, CHX caused erythrocytes and neutrophils lysis [36 37], was able to inhibit the protein synthesis [11 38] and reduced the adhesion and production of matrix components in fibroblasts [39 40 41]. Furthermore, Eren et al. [16] found that CHX used as a mouthwash was able to induce DNA changes in oral epithelial cells and lymphocytes.

Na-DNA is able to pass through the cell membrane by pinocytosis and acts as a donor of purine and pyrimidine bases, which are key molecules for cell vitality [17]. Previous studies showed that this compound could stimulate the growth of osteoblasts, fibroblasts and other cell lines *in vitro* [19 20 42 43 44] demonstrated that nucleotides and nucleosides operate on cell proliferation in two different ways: they can stimulate nucleic acid synthesis through the salvage pathways [45 46], and they can bind and activate purinergic receptors [20 21 46].

Unsurprisingly, in a short observation period, such as in the present study, no effect of Na-DNA on controls could be observed. On the contrary, Na-DNA was more effective in suffering cells, both after CHX treatment and induction. The repairing activity of Na-DNA, as evidenced in the previously cited studies, can provide an explanation for this finding. Furthermore, histological findings of this study showed an enlargement of extracellular spaces in outer layers after oxidative stress induction that increased the penetration of the active principles contained inside the mouthwashes. In this way, we might explain the better morphological aspects of the innermost cell layers. It has to be observed that, contrary to *in vivo* situations, the basal layer of ROE specimens was freely exposed to the external environment via the artificial basal membrane. The effects of

oxidative stress induction were therefore observed on both the outer and inner sides of ROE specimens.

It is widely known that the human oral mucosa is made of an outermost layer of stratified squamous epithelium, which is similar to stratified squamous epithelia found in the rest of the body. It has a mitotically active basal cell layer, many differentiating intermediate layers, and a superficial layer, where cells exfoliate from the surface of the epithelium. Stratified, cultured TR146 cell layers are derived from a human neck metastasis originating from a buccal carcinoma. They can be used to create an *in vitro* model of the reconstituted human oral epithelium (ROE) where the stratified epithelium that is formed closely resembles normal human buccal epithelium [47]. As the oral epithelial tissues are reconstituted in a physiologically natural environment and on a chemically defined medium, they express all major natural markers of the basal epithelial layer and behave as in vivo human epithelial cells when treated with pharmacologically active or cytotoxic compounds. The model also exhibits tissue repair mechanisms that reflect the in vivo wound healing processes [47] [48] [49]. For these reasons using ROE as an alternative to human and animal testing of drugs allows overcoming many concerns about the activity of newly developed active principles as well as ethical concerns.

Data regarding the activity of Na-DNA mainly derive from in vitro studies performed on monolayer cultures experiments [21] [22] [23] [44] [50]. This kind of experimental setup is generally recognized as a sensitive method to test the regenerative potential of a compound after a cytotoxic challenge [51] [52]. However, monolayer cell culture models cannot give a realistic approach to the complex chemical and physical microenvironment of natural tissues. In addition to that, tissue cells are in constant communication with each other, modulating their behavior based on changes in the surrounding microenvironment [53] [54]. From this point of view, an approach based on a bioreactor and ROE provides many advantages compared to the other *in vitro* models.

The model applied in the present study allows performing studies on drug effects with precise measurements under controlled conditions that are very similar to *in vivo* ones. Nevertheless, *in vivo* studies are still necessary as *in vitro* simulations can, no matter how accurately, reproduce only parts of the very complex interactions taking place in the oral environment. It is a balancing act between the speed, cost, and purpose of the model that dictates its selection and use.

The results of the present study demonstrate that Na-DNA showed a clear and protective action against cellular damage due to oxidative stress and, partly, to exposure to the CHX-containing mouthwash solutions. CHX showed a cytotoxic effect that was mitigated by the association with Na-DNA. It may be speculated that this result could be due to a concurrent repairing activity expressed by Na-DNA.

These results open the possibility to test the protective activity of Na-DNA in association with other compounds that are known to provide adverse effects to oral soft tissues, such as, for instance, sodium dodecyl sulfate, which is contained in most of the oral care products. Further studies may also test the association of Na-DNA with other ones having similar activity, such as, for instance, hyaluronic acid or α-tocopherol, evaluating possible synergistic effects or improving the activity of already existing formulations.

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