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The Evaluation of Sodium Lauryl Sulphate in Toothpaste on Toxicity on Human Gingiva and Mucosa: A 3D *in vitro* Model

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Abstract

Five different commercial toothpaste formulations were labeled as A, B, C, D and E and evaluated for toxicity *in vitro* using reconstituted human oral and gingival mucosa cultures. Toothpastes A, D and E contained sodium lauryl sulphate (SLS) while toothpastes B and C did not. All toothpastes contained fluoride in different quantities. Tissue viability (MTT), tissue morphology (LM and TEM) and release of pro-inflammatory mediator IL-1α were evaluated. For gingival mucosa, topical exposure of toothpastes did not affect MTT values. Examination of oral mucosa however, showed that toothpastes B, E and D induced a significant loss of viability after 1 h (49.2%, 55.5% and 78.4% respectively) ($p<0.05$). After 3 hours toothpaste B demonstrated a 51.4% loss of viability whereas tissue viability for toothpastes D and E dropped to 11.1% and 4.5% respectively. The release of pro-inflammatory mediator IL-1α showed that only gingival mucosa tissues exposed to toothpaste D and E showed a marked increase of IL-1α after 1 hour, and toothpaste A, D and E after 3 h exposure. Oral mucosa tissue exposed for 1 h resulted in increased levels of IL-1α for toothpastes A, B, D and E, which became more important at 3 h. Morphological analysis of the oral mucosa demonstrated partial necrosis after exposure to toothpastes A, B and C, and severe necrosis to D and E. MTT cannot be used as single toxicity parameter and should be confirmed by histology. Both in vitro oral and gingival mucosa models are suitable to evaluate the toxicity of toothpaste formulations. The presence of SLS in toothpaste formulations is presumably responsible for the toxicity observed *in vitro*. MTT, IL-1α release and morphology were affected by the SLS containing toothpastes A, D and E. These observations confirm clinical inflammatory effects of SLS in oral care products often reported in literature.

Keywords: Oral mucosa; Gingival mucosa; Toothpaste; Fluoride; MTT; 3D cell cultures; Toxicity; Sodium lauryl sulphate

Introduction

Maintaining good oral hygiene is a very important issue in everyday life and especially during an orthodontic treatment. The term 'oral hygiene products' is recent but there is historical evidence dating back at least 6000 years that formulations and recipes existed to benefit oral and dental health [1]. Throughout centuries, most toothpowders, toothpastes and mouth rinses appeared to have been formulated for cosmetic reasons including tooth cleaning and breathe freshening rather than the control of dental and periodontal diseases [2]. During the last century, toothpastes became less abrasive [3] and a set of safety standards were developed [4-6].

By virtue of common usage, toothpastes are the ideal vehicles for plaque control agents. A number of ingredients are being used to formulate toothpaste and each has a role in either influencing the consistency and stability of the product or its function [7,8]. These ingredients include abrasives, detergents, thickeners, sweeteners, humectants, flavours and active ingredients [9,10]. The most common detergent used in toothpaste is the anionic compound sodium lauryl sulfate (SLS), which imparts the foam and 'feel' properties to the product. Additionally, detergents may help dissolve active ingredients and SLS has both antimicrobial and plaque inhibitory properties [11]. In fact SLS was shown to have moderate substantivity measured at between 5 and 7 hours and plaque inhibitory action. However SLS also induces prominent changes in the histological structure of animal cheek pouch epithelium. These changes include hyperkeratosis, hypergranulosis, acanthosis and varying degrees of basal hyperplasia, all of which result in a significant increase in epithelial thickness [12]. The known untoward side effects of SLS in the human oral cavity are inflammation and desquamation of oral mucosa [13-15].

The aim of the present study was to investigate whether the presence of SLS in marketed toothpastes would alter the morphology and biochemical properties (viability and inflammatory cytokine release) of human 3D cell cultures. Since tissue cell cultures are generally recognised in the literature as being a sensitive method of assaying the cytotoxic potential of test materials [16-18], the objective of this experiment was to measure the toxicity of 5 marketed toothpastes on a reconstituted human oral and gingival epithelium model. The working hypothesis was that toothpastes containing SLS showed more negative effects on cell cultures.

Materials and Methods

A complete synopsis of the experimental protocol is shown as flowchart in Figure 1.

Test materials

Toothpastes were blinded and labelled as A, B, C, D and E (Table 1). Toothpastes A, D and E contained SLS while toothpastes B and C did not. They all did contain fluoride. A detailed listing of the ingredients in each toothpaste is listed in Table 1. In order to simulate the oral condition (dilution by saliva), toothpastes were mixed with water in 1:2 ratios to produce a 30% toothpaste slurry. As positive control, SLS

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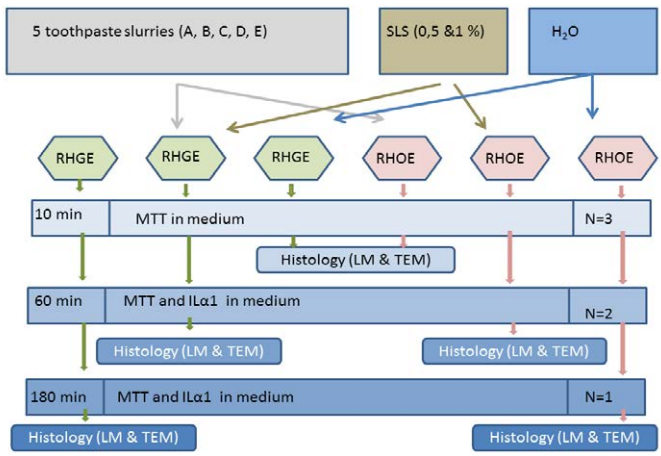


Figure 1: Synopsis of Experimental Protocol.

Code	Toothpaste	Ingredients
A	Thera-med Original Batch number: 0520741 Schwartzkopf and Henkel, Dusseldorf, imp. B-1080 Brussels, Nieuwegein	Aqua, Hydrated Silica, Glycerin, Sorbitol, PEG-32, Sodium lauryl sulfate, Aroma, Cellulose gum, Trisodium phosphate, Disodium phosphate, Sodium saccharin, Zinc sulfate, Sodium benzoate, CI 14720, CI 42090, CI 69800, CI 42090 . Contains Sodium fluoride (1450 ppm F^-)
B	Elmex Batch number: 4385G2 Johnson and Johnson, GABA B.V., Postbus 1039, 1039 BA, Almere	Aqua, Hydrated Silica, Sorbitol, Hydroxy ethyl cellulose, Aroma, Titanium dioxide, Saccharin Contains Olafluor (1250 ppm F^-),
C	Zendium Classic Batch number: 125111; 152301; 7304742 Kortman intradal SA, Sara Lee Hand BC, Belgium, 1702 Groot-Bijgaarden	Aqua, Hydrated silica, Glycerin, Sorbitol, Steareth-30, Carrageenan, Aroma, Titanium dioxide, Disodium phosphate, Citric acid, Sodium benzoate, Sodium saccharin, Potassium thiocyanate, Glucose oxidase, Amyloglucosidase, Lactoperoxidase Contains Sodium fluoride (1100 ppm F^-)
D	Signal Integral Batch number: 405671WB Unilever, Lever Fabergé Belgium, Unibel N.V., 1190 Vorst	Aqua, Hydrated Silica, Sorbitol, Calcium Carbonate, Sodium Lauyl Sulfate, Sodium Monofluorphosphate, Aroma, Sodium Fluoride, PEG-32, Cellulose Gum, Methylparaben, Propylparaben, CL 73360 Contains eucalyptus-extracts and Sodium fluoride (1450 ppm F^-)
E	Colgate Blue Minty Gel Batch number: 4146IT10305 Colgate-Palmolive, 1160 Brussel, 4041 Liege	Aqua, Hydrated Silica, Glycerin, Sorbitol, PEG-12, Sodium lauryl sulfate, Aroma, Cellulose gum, Sodium saccharin, Dicalcium phosphate dihydrate, CI 42090, CI 42090 Contains Sodium fluoride 0.1% (450 ppm F^-), Sodium monofluorophosphate 0.76% (1000 ppm F^-)

Table 1: Ingredients of the tested toothpastes (as mentioned by manufacturers).

solutions (0.5 and 1% w/v in water) were used and water is used as negative control.

Tissue culture models and experimental treatment

Commercially available 0.5 cm^2 inserts containing reconstituted human oral epithelium (RHOE) and reconstituted human gingival epithelium (RHGE) were obtained by culturing either human oral epithelial cells (TR-146 cell line of oral squamous carcinoma) or human gingival cells (primary cells from a gingival epithelial biopsy) on polycarbonate culture inserts lifted to the air-liquid interface for 7 days (SkinEthic Laboratories, Nice, France). Prior to testing, the RHOE and RHGE inserts were placed onto 300 µl of maintenance medium. 30 µl of a 30% dilution of each toothpaste product (A, B, C, D and E) was applied onto triplicate cultures which were incubated at 37°C, 5% CO_2 for 10 minutes, 1 h and 3 h. 30 µl of water (negative control) was applied in parallel onto triplicate cultures and 30 µl of a 0.5 or 1% SLS solution was applied as positive controls, also in triplicate. For each condition, at the end of the test period, duplicate cultures were placed into appropriated labelled wells for MTT cytotoxicity testing. Additionally, the medium underneath each culture was collected and stored at -20° C for extracellular release of pro-inflammatory mediators IL-1α.

MTT assay procedure

The MTT assay was performed by placing the treated 0.5 cm^2 inserts containing RHOE and RHGE in wells containing 0.3 ml of 0.5 mg/ml MTT solution (Sigma-Aldrich St. Louis, MO, USA solution lot no. 02010AD0602). All tissues were then incubated at 37°C for 3 h. For qualitative evaluation of cell viability, after 30 minutes incubation, the colour of each culture was noted. Negative control cultures have to be dark blue colour, proof of cell's viability. Positive control cultures have to be blue/white, evidence of cell death. For quantitative evaluation of cell viability extraction was performed at room temperature in 1.5 ml of isopropanol for a minimum of 1.5 h, by gentle shaking. 200 ml of extracts were transferred in a 96 well plate and OD was measured at 570 nm by a spectrophotometer (Dynex Technology, Chantilly, Virginia USA). Reference filter: 690 nm.

$$\% \text{ of viability} = (DO_{570} \text{ nm sample} / DO_{690} \text{ nm control}) \times 100.$$

IL-1α quantification measurements

The amount of pro-inflammatory mediator IL-1α was quantified in the medium underneath the RHOE and RHGE tissues by quantitative sandwich immunoassay technique (Quantikine Human IL-1α, R&D Systems, UK, Ref DLA50, lot 207423). After topical application, the cultures were incubated on 0.3 ml of defined medium for 1 h and 3 h. Conditioned media were collected and kept frozen for cytokine quantification. The inflammatory mediators released in these conditioned media were then quantified using ELISA kits, specifically for each type of mediator to be measured. For each sample and standard, 200 µl of the collected medium was incubated in the pre-coated 96-well plate wells for 2 hours at room temperature. After washing, 200 µl of IL-1α conjugate was added and the plate was consequently incubated for 60 minutes. After incubation, all wells were washed 3 times and 200 µl of TMB/H_2O_2 was added. Following incubation for 20 minutes, the reaction was stopped by adding 50 µl of 2N H_2SO_4. All wells were consequently read in a spectrophotometer (Dynex Technology, Chantilly, Virginia, USA) at 450 nm (ref filter 570 nm). The concentrations of IL-1α present in the samples were calculated on the basis of a calibration curve. In all cases, duplicate measurements were performed for each sample.

Morphology evaluation by light and electron microscopy

All remaining manipulations were hereafter performed at room temperature. For each condition, at the end of the test period, one of the triplicate cultures was cut in half. One moiety of the tissue was fixed in a 10% formalin solution and later embedded in paraffin. Four micron vertical sections were stained with hematoxy/eosin and photographed under a light microscope. The sections were evaluated with the light

microscope Leica® (DMR®, Wetzlar, Germany) at 10x, 20x, 40x and 63x magnification.

For Transmission Electron microscopy (TEM) (Tecnai 10 Philips®, Eindhoven, The Netherlands), the second half of the tissues were fixed with a solution containing 1.5% glutaraldehyde in cacodylate buffer 0.1 M and sucrose 0.1 M (pH 7.4) at 4°C. After 30 minutes treatment, the solution was removed and 3 ml of osmium tetroxide (OsO_4) 1% in 0.1 M cacodylate buffer was added at 4°C [19]. 1 h later the cultures were rinsed three times with distilled water. All remaining manipulations were thereafter performed at room temperature. The tissues were stained with 3.5 % of uranyl acetate in water for 10 minutes. After three short rinses with distilled water, followed by a dehydratation in an alcohol series, dehydratation was stopped by transferring the tissues in pure ethanol two times for 10 minutes, followed by one time for 20 minutes. The final step consisted of dipping the biopsies and cell cultures into a mixture of ethanol 100° with Epon resin (Polysciences, Eppelheim, Germany) 1/1 for 60 minutes. The biopsies and cell cultures were consequently immersed overnight in Epon at room temperature and the next day, the filter was carefully cut out, imbedded in fresh Epon and cured in the oven at 60°C for 2 days. Sections were made with an ultramicrotone Ultracut (Reichert®, Wien, Austria).

Statistical analysis

The effect of the different treatments and the treatment period on the viability and the IL-1α release for both the RHOE and RHGE were assessed with a two-way ANOVA. The normality of the residuals was analysed with a Kolmogorov-Smirnov test and the homogeneity of the variances was tested with the Levene's test. When the variances were found not to be equal the data were log-transformed. To further compare the difference between the treatments for each time period and to evaluate the time effect for each treatment a Bonferoni post hoc test with a significance level of $p<0.05$ was used. The statistical analysis was performed using SPSS version 12.0.

Results

Evaluation of cell viability (MTT test)

The influence of the toothpastes on the viability of the RHOE and RHGE in function of time is shown in Table 2. For gingival mucosa, topical exposure of toothpastes did not affect MTT values. Examination of oral mucosa however, showed that the toothpastes B, E and D induced a significant loss of viability after 1 h (respectively 49.2%, 55.5% and 78.4%, $p<0.05$). After 3 h, toothpaste B demonstrated a loss of viability of 51.4 %, whereas tissue viability for toothpaste D and E dropped to 11.1% and 4.5% respectively. For the RHGE all the toothpastes showed a viability that was statistically comparable with the negative controls after a 3 h treatment. However, for the RHOE, toothpastes B, D and E induced a decrease of the viability after 1 h and for toothpastes D and E longer treatment resulted in a further decrease of the viability that was statistically significant from the negative control after 3 h. Toothpastes A and C did not affect the viability of the RHOE, after 3 h the viability was still comparable with the negative control. For both the RHOE and RHGE the positive controls (SLS 0.5% and 1%) resulted in a time and concentration dependent decrease of the viability.

For both models all negative control treated tissues were coloured blue and all positive control treated tissues coloured white after 1 hour. All tested toothpastes showed a statistically different toxicity profile for each tissue type ($p<0.001$).

Release of inflammatory mediator IL-1α

Table 3 shows the release of the inflammatory mediator IL-1α for the different treatments in function of time. For the RHOE toothpastes A, B, D and E induced a significantly increase of IL-1α after 60 minutes when compared to the negative control. The IL-1α release increased significantly for toothpastes A, C, D and E although for toothpaste C the release after 3 h was statistically lower when compared to the other toothpastes and to the positive controls. Toothpastes B and C did not affect the IL-1α release of the RHGE, after 3 h the levels were

Treatment	Viability MTT (%)[1]		
	10 min	60 min	180 min
RHOE			
Control	100.0 ± 1.4[a]	100.0 ± 0.6[a, b]	100.0 ± 2.7[a, b]
SLS 0.5%*	93.2 ± 2.3[a]	44.9 ± 6.4[c, d]	29.4 ± 3.7[c]
SLS 1%*	81.4 ± 3.4[a]	30.3 ± 0.9[d]	3.5 ± 1.7[e]
A	109.5 ± 3.7[a]	106.4 ± 5.4[a]	103.1 ± 14.4[a]
B*	104.7 ± 2.6[a]	49.2 ± 3.1[b, c, d]	51.4 ± 21.1[b, c]
C	109.3 ± 2.1[a]	92.2 ± 1.3[a, b]	95.9 ± 1.5[a, b]
D*	121.8 ± 9.8[a]	78.4 ± 35.4[a, b, c]	11.1 ± 5.1[d]
E*	110.8 ± 6.9[a]	55.6 ± 10.3[a, b, c, d]	4.5 ± 0.4[e]
RHGE			
Control	100.0 ± 1.8[a, b, c]	100.0 ± 6.9[a]	100.0 ± 3.2[a]
SLS 0.5%*	87.4 ± 5.7[b, c]	40.8 ± 0.8[b]	33.1 ± 1.5[b]
SLS 1%*	82.8 ± 1.4[c]	28.0 ± 4.1[c]	4.6 ± 0.2[c]
A	100.4 ± 3.2[a, b, c]	98.9 ± 6.0[a]	102.4 ± 2.8[a]
B	103.2 ± 3.0[a, b]	103.3 ± 1.7[a]	95.9 ± 6.1[a]
C	97.8 ± 4.7[a, b, c]	96.5 ± 2.7[a]	106.2 ± 14.6[a]
D	101.9 ± 5.2[a, b, c]	98.1 ± 0.7[a]	110.3 ± 2.8[a]
E	108.5 ± 0.3[a]	102.1 ± 7.4[a]	100.4 ± 10.4[a]

[1]Values represent the mean ± SD, n=2
[a,b,c,d,e]Within each time period means with the same superscript are not significantly different from each other.
*This treatment resulted in a significant decrease in viability in function of time

Table 2: Influence of the different toothpastes on the viability in function of time.

Treatment	IL-1α[1]	
	60 min	180 min
RHOE		
Control	1.0 ± 0.0[a]	1.0 ± 0.0[a]
SLS 0.5%*	36.0 ± 3.9[c]	66.6 ± 7.2[d]
SLS 1%*	27.6 ± 10.8[c]	63.3 ± 7.1[d]
A*	8.0 ± 0.4[b]	23.9 ± 2.6[c]
B	27.7 ± 12.9[c]	25.1 ± 7.7[c]
C*	1.1 ± 0.3[a]	9.0 ± 3.3[b]
D*	11.7 ± 2.2[b]	39.4 ± 2.0[c, d]
E*	8.4 ± 3.5[b]	35.2 ± 7.9[c, d]
RHGE		
Control	1.1 ± 0.2[a]	1.3 ± 0.1[a]
SLS 0.5%	50.5 ± 21.5[b]	68.9 ± 14.1[c]
SLS 1%	34.6 ± 19.1[b]	65.5 ± 9.7[c]
A*	3.2 ± 1.7[a]	15.0 ± 4.0[b]
B	3.0 ± 2.7[a]	2.1 ± 0.7[a]
C	2.6 ± 1.2[a]	1.5 ± 0.1[a]
D	18.7 ± 12.3[b]	28.5 ± 13.8[b, c]
E	28.7 ± 18.3[b]	57.4 ± 13.7[b, c]

[1]Values represent the mean ± SD, n=3
[a,b,c,d]Within each time period means with the same superscript are not significantly different from each other.
*This treatment resulted in a significant increase of IL-1**α** in function of time

Table 3: Influence of the different toothpastes on the IL-1α release in function of time.

still comparable with the negative control. Toothpaste A induced a significant increase after 3 h, whereas toothpaste D and E resulted in a significant increase after 1 h when compared to the negative controls. The positive controls induced a significantly increase of IL-1α after 1 h in comparison with the negative control for both the RHOE and RHGE. Cell type was not significant but showed different results for different tested products.

Histology

For each test material, at the end of each test period, half tissue was fixed in a 10% balanced formalin solution and embedded in paraffin for LM, the other half in Epon for TEM. The histopathological interpretation was peered by 2 patho-anatomologist who came to the same conclusions ($p<0.05$). Histopathological examination of LM of the *in vitro* oral and gingival epithelium model is classified as follows [20-22]:

- No irritation (NI): constant thickness of the epithelium, regular and compact shape, cells attached to the others;
- Mild irritation (MiI): minimal changes with slight oedema;
- Moderate irritation (MoI): beginning spongiosis in upper layers and architectural atrophy, cellular irregularity;
- Severe irritation (SI): disintegration of the upper cell-layers, spongiosis, cellular necrosis, loss of cellular junction in basic layer.

The results of both cell cultures are presented as colour slides. Negative control scored NI (T1-T3), Positive control SI (T1-T3) as well as toothpastes D and E (Figure 2) on RHOE. MiI is seen for tooth paste A, B and C after 1 h partial necrosis in all toothpastes on RHOE. The results of TEM showed similar effects with severe disturbances at ultrastructural level of toothpaste A, D and E after 1 h and 3 h both on RHOE and RHGE (Figure 3). Apoptosis and necrosis is observed after 1 hour and 3 hours in the latter toothpaste samples (Figure 3).

Discussion

Vacuolisation was reported in previous studies on monolayer and confirms the histological results in the present study [13].

The 5 marketed toothpastes were evaluated blind. A 30% solution was chosen in order to simulate normal use of toothpaste as half of the amount is washed or rinsed after the first application. The amount of 30% dilution was considered as the actual used portion *in vivo*. It is important to note that, with saliva in the mouth, the final dilution of toothpaste encountered during brushing is about one-third [23]. All toothpastes contained a fluoride formulation. However, the effect of fluoride was not considered although previous *in vitro* studies indicated that sodium fluoride can be toxic to oral mucosal fibroblasts *in vitro* by its inhibition of protein synthesis, mitochondrial function and depletion of cellular ATP [24,25]. Using the gingival epithelial tissues, only an effect was noticed at the level of IL-1α release. No toxicity was measured with MTT. Based on this end point, the toothpastes could by ranked in order of increasing irritation potency as follows:

C (Zendium) = B (Elmex) < A (Thera-Med) < D (Signal) < E (Colgate)

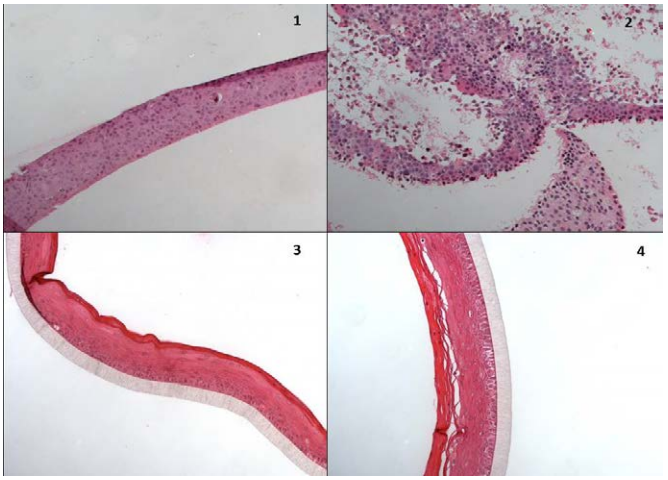
Using the oral epithelial tissues an effect of the toothpastes was noticed on the viability, the IL-1α release (less discriminative) and on the histology. Based on the different endpoints the following rank order of increasing irritation potency could be established:

C (Zendium) < A (Thera-Med)* < B (Elmex) < D (Signal) ~ E (Colgate)

*For Thera-Med high viability was observed after 3 h, however the tissue was described as necrotic.

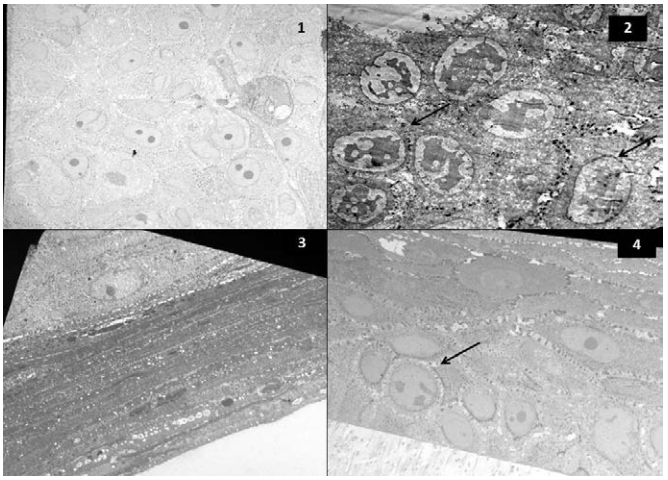
Conclusion

Based on the histological findings and the statistical interpretation of the quantitative evaluation of the biochemical endpoints, the following conclusions could be drawn. The RHOE model is more sensitive compared to RHGE. This is due to the morphological differences in the tissue differentiation which is far more advanced in the RHGE, featuring the presence of a thin stratum corneum, little parakeratosis and few or no granular cell layers, opposite to the less differentiated RHOE. False negative results are obtained when tissue viability (MTT assay) is being used as a single end point to evaluate



Notice the dramatic effects of toothpaste E on RHOE.

Figure 2: Light microscopy (x40) effects of toothpaste E on RHOE (2) severe irritant (SI) and RHGE (4) severe irritant (SI) compared to non-treated cultures RHOE (1) (NI) and RHGE (3) (NI) both at 180 min.



Notice the dramatic effects of toothpaste E on RHOE on ultrastructural level marked with necrotic and apoptotic cells (arrows).

Figure 3: TEM (x6200) effects of toothpaste E on RHOE (2) and RHGE (4) compared to non-treated cultures RHOE (1) and RHGE (3) both at 180 min.

the toxicity potential of toothpaste formulations. Simultaneous histological evaluation is therefore mandatory for accurate toxicity assessment. Toothpastes containing SLS react different. This was clearly demonstrated for all endpoints. In order to validate the assay as a valid alternative to animal models, other substances have to be evaluated to substantiate the predictive value of both tissue models and experimental protocols used.

Clinical Relevance

Based on the findings of the study, we can make following recommendations. Usage of large amount of toothpaste sould be avoided (especially in children), toothpastes containing SLS are not intended to stay in the mouth and proper rinsing after brushing is advised, patients sensible to aphtuous laesions should be informed not to use toothpastes containing SLS.

Further research is needed to explain the appearance of apoptosis in these cell cultures after topical toothpaste application.

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