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Toothpaste detergents: a potential source of oral soft tissue damage?

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Abstract: *Objectives:* Toothpastes are thought to be of benefit to cleaning teeth but may also have the potential for soft tissue damage at least on the cellular level by inclusion of detergents in their formulation. The aim of this study was to observe the *in vitro* response of oral mucosa like cells to toothpaste detergents. *Methods:* TERT-1 keratinocytes were exposed to varying concentrations of the detergents Adinol, Sodium Lauryl Sulphate, Tego Betain and Pluronic as well as PBS and culture medium. After 2-min exposure, cells were washed and incubated in fresh medium for 24 h. Cell death was then spectrophotmetrically measured using an MTT assay. *Results:* Except for Pluronic, cell viability was markedly reduced for all detergents at all increasing concentrations when compared to the positive medium control. Cells treated with Pluronic were stimulated compared to medium alone. *Conclusions:* These *in vitro* data suggest that some detergents may have the potential to cause soft tissue damage in the mouth. Although *in vivo*, saliva may neutralize such effects. The results for Pluronic suggest a possible oxidative stress response that bears further study.

Key words: detergent; MTT; oral mucosa; toothpaste; viability

Introduction

Toothpastes are formulated to benefit oral care, but there is the potential for harm to both hard and soft tissues mainly from contained abrasives and detergents (1). A review on the subject concluded that in normal use toothpastes produce clinically insignificant effects to hard tissue; pointing out however, that little is known of the effects on soft tissues (2). Detergents routinely added to toothpastes for their foaming

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properties provide moderate antibacterial and plaque inhibitory properties (3, 4). The most common detergent, SLS, can induce irritant dermatitis (5–7). The irritant effect of surfactants is an increasing area for concern due to common use in cosmetics and pharmaceutical products (8) and could be relevant to the oral mucosa, particularly related to the effects of increased inflammation leading to exacerbation of periodontal disease, or increasing the risk of infection leading to periodontal disease. The irritant effect of detergents could also be relevant to initiation and progression of gingival recession and recurrent oral ulceration. Moreover, it has been suggested that SLS could exacerbate conditions with compromised epithelial integrity (22–24). Given the amount of inflammatory activity in the mouth of a patient with recurrent oral ulceration however, it cannot be categorically concluded that the small quantities of SLS wrapped up in a toothpaste formulation were not having some effect on the surrounding healthy tissue. Nevertheless, it would be useful to determine which detergents currently in use in toothpaste formulations, present the greatest threat to cellular viability and the structural integrity of the gingival tissue.

The aim of this *in vitro* study was to investigate the effects of four toothpaste detergents on oral mucosa-like cells. Cell viability or death was measured using an MTT assay which measures the ability of live cells to metabolize yellow MTT into purple formazan.

Methods and materials

Culture of immortalized normal oral (TERT-1) cells

TERT-1 cells were chosen as the analogue to oral mucosa cells because the cell line OKF6/TERT-1 represents normal oral mucosal epithelium (floor of the mouth) immortalized by forced expression of telomerase via retroviral transduction (17). The OKF6-TERT-1 cell line was grown in keratinocyte serum-free medium (KSFM; Invitrogen) containing 25 μg ml^{-1} bovine pituitary extract, 0.4 ng ml^{-1} epidermal growth factor (EGF) [diluted in 20 mM HEPES-buffered Earle's salt, 0.1% bovine serum albumin (BSA)] and the calcium chloride ($CaCl_2$) concentration increased to 0.4 mM. The culture medium was replaced every 2 days (21).

Every 7–10 days, cells were subcultured when ~90% confluent. The culture medium was discarded and the cultures were washed with PBS. The ells were enzymatically detached by incubating in 0.025% (v/v) trypsin/0.01% (w/v) EDTA solution in PBS for 5–30 min at 37°C. The trypsin/EDTA solution was neutralized by the addition of an equal volume of epithelial medium and the cell suspension was centrifuged (60 *g*, 10 min). The pellet was resuspended in 5 ml of culture medium. Cell number was determined using coulter counter (Coulter Z Series, Coulter Corporation, USA). Cells were re-seeded at 5×10^5 cells per 75 cm^2 flask in epithelial medium and maintained in culture, as previously described.

Stocks of TERT-1 keratinocytes were maintained in liquid N_2. After determination of the cell number, 5×10^5 – epithelial medium with 20% (v/v) fetal bovine serum (FBS) and 20% dimethylsulphoxide (DMSO) was added to 1×10^6 cells suspended in KSFM medium prior to freezing and then transferred into cryovials. The cells were placed in a −70°C freezer for 12–24 h before being transferred to liquid N_2 at −196°C for long term storage.

Cells were recovered from storage in liquid N_2 by rapid thawing of the cryovials in a water bath at 37°C. The cells were then transferred into 10 ml of epithelial medium and centrifuged (60 *g*, 10 min). The supernatant was discarded, the cell pellet resuspended in epithelial medium for tumour-derived cell lines or KSFM medium for the OKF6/TERT-1 cell line and the suspension transferred to a 75 cm^2 tissue culture flask. The cells were maintained in culture, as previously described.

Detergent exposure

Cultured cells from 75 cm^2 tissue culture flasks were seeded out in 1 ml aliquots into 24 well culture plates and left to grow until 70–90% confluent, medium being replaced every 2 days as previously described. At optimal confluence, medium was removed from wells, and replaced with 1 ml of each concentration of each detergent in triplicate. Three wells were also allocated for fresh medium, and three for PBS as positive and negative controls. The concentrations of detergents were made up at 1%, 0.5% and 0.25% using PBS sterilized using a 0.2 μm filter, with the exception of Tego Betain, which was made up at concentrations of 0.325%, 0.162%, and 0.081% based on ratios of concentrations recommended by suppliers. These concentrations are considered to be those that are present in a whole toothpaste formulation, before dilution with saliva occurs in the mouth during use. All controls and detergents were warmed in a water bath at 37°C prior to application. Detergents used were SLS, Adinol, Tego Betain, and Pluronic. Exposure time was for 2 min (comparable to toothpaste exposure time *in situ*).

MTT assay

Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means.

After exposure to detergents or controls, wells were aspirated and fresh medium added for incubation for 24 h. MTT (Sigma, St. Louis, MO, USA) was made up as a 5 mg ml^{-1} stock in warm serum free medium and left in the dark for 20 min to dissolve. The solution was filter sterilized using a 0.2 μm filter and diluted in KSFM medium to give a 1 mg ml^{-1} solution.

Following incubation, wells were aspirated once more, and 1 ml of the MTT solution added to each well and incubated for 1 h. The MTT solution was removed, the cells rinsed with PBS and the resulting water-insoluble formazan salt, created by the metabolic activity of viable cells, was dissolved using 1 ml per well DMSO. The absorbance of the salt solution at 520 nm was determined with DMSO as a blank.

Replication

The above methods were carried out three times using three separate batches of TERT-1 keratinocytes retrieved from cold storage. In each case, all treatments were also performed in triplicate by treating three wells per detergent/control. This was to ensure reproducibility.

Statistical analysis

Because the sample number for each experiment (replication) was limited to three wells per treatment type, the data were assumed to be normally distributed. Descriptive statistics showed standard deviations that suggested normal distribution to be highly likely. An ANOVA was chosen for statistical analysis due to the number of treatment groups to be compared. In addition a two way ANOVA was carried out for each concentration on all detergents types as there were essentially two determining factors within this study; detergent type, and detergent concentration. It was therefore of interest to see how a lower concentration of one detergent type compares to a higher concentration of a different detergent type as well as to differing concentrations of the same type. These tests were performed using Microsoft Excel™ as the complexity of the data was such that use of a more powerful statistical package was not necessary.

Results

The absorbencies from MTT metabolism by viable cells after exposure to detergents of varying concentration, PBS, or Media are shown in Fig. 1. The depth of colour in each well observationally signified the number of viable cells after exposure, those wells with the least colour having experienced cell death by apoptosis, necrosis or cell lysis. In observation, three out of the four detergents caused almost total cell death after

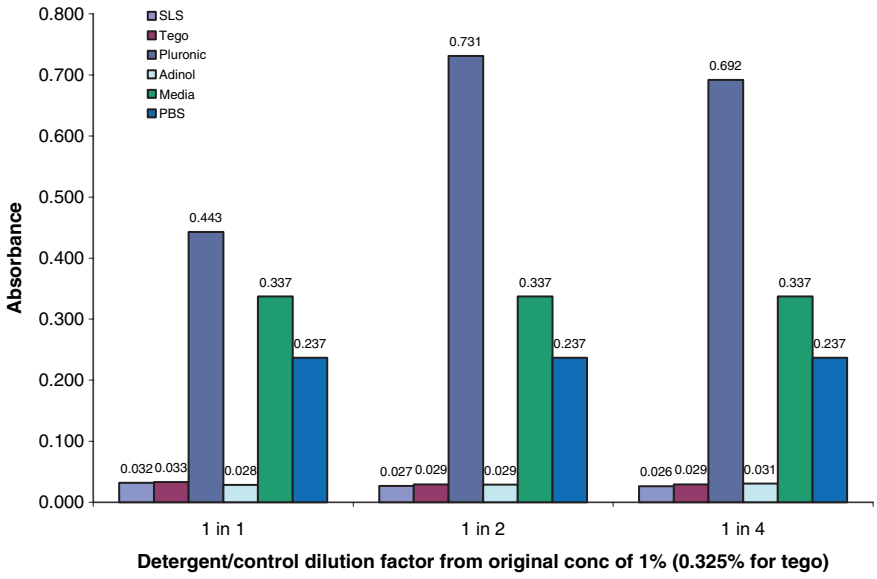

Fig. 1. Change in viability of cells with varying concentration of detergent/control.

2 min; absorbance rarely increasing beyond 0.03. Concentration appeared to have no effect on the level of cell death, as the means absorbencies of the three triplicate experiments were similar for all three concentrations and for all three detergents that adversely affected the cells. It could be argued that Tego Betain caused least cell death as the three mean absorbencies remain at 0.029 and above, whereas the other two (SLS and Adinol) were generally around the 0.027 order of magnitude. Media as the positive control showed much higher cellular viability than the three harmful detergents at their three concentrations. Pluronic however, actually revealed higher cellular viability than even fresh media, with an average absorbance of 0.443 at 1%. It is at this point that concentration became a factor, with absorbance (and therefore cellular viability) increasing as concentration decreases. Of particular note here is that the concentration of Tego Betain at its highest was greater than that of Pluronic at its lowest. Despite this, Tego Betain exposure at even low concentrations resulted in very low cellular viability compared to Pluronic at similar concentrations. Two way analysis of variance revealed a very high statistical significance between detergent types at a dilution factor of 1 ($P = 5.75 \times 10^{-16}$). Significance decreased slightly with dilution, with *P*-value at 1 in 2 dilution from the original concentration of 2.27×10^{-15}, and at $P = 5.88 \times 10^{-13}$ at dilution factor 1 in 4.

Discussion

In the present study three out of the four detergents, at all concentrations, caused cell death. Notably, the concentration of Tego Betain at its highest was almost equal to that of the other detergents at their lowest and yet the absorbencies were very similar. It appears that even at 0.25% (or 0.0812% for Tego Betain); cellular viability is reduced to the same levels as 1% (0.325% for Tego Betain). This indicates that any exposure at any level to these particular detergents causes some form of cell death, though it is unclear at this stage whether this is due to necrosis or programmed cell death (apoptosis). Detergent concentration, though shown here to illicit the same effects regardless of concentration, may be a factor at much lower concentrations than used here or in toothpaste manufacture. However it is unlikely that much lower concentrations could be used in toothpaste manufacture without compromising their basic function. Given the similarity of reduction in cell number with the three harmful detergents at all concentrations it is likely that the cells reached a saturation point relatively quickly, and cell death was caused early on thereby giving the impression that all concentrations affected the cells in the same way. It is probable that the ionic properties of the detergents were directly responsible for initiating cell death, and with this information it is possible to theorize the likely mechanism of the cell death seen here (14). The mean absorbance for the control experiments of exposure to either media or PBS showed a great deal of variation, particularly in experiment two where the value for both media and PBS are much lower than either of the two other repeats. It is possible that this result is due to a contaminant introduced unknowingly during treatment, though this effect is not seen in any of the detergent treatment groups which were performed at the same time. This result being confined to one experiment rather than being seen for media in experiment one and for PBS in experiment two is encouraging however in terms of accuracy of the test groups in experiment two and of the two unaffected experiments.

The results for Pluronic are somewhat surprising as this detergent actually appeared to increase cell viability compared to treatment with media alone. Pluronic is one of a range of detergents that are triblock copolymers of ethylene oxide and propylene oxide and these copolymers are high-volume reactive alkylating agents. Farooqi *et al.* (15) reported that these agents induced chromosomal aberrations, micronuclei and sister-chromatid exchanges in mouse bone marrow cells when injected intraperitoneally. They were, in essence, genotoxic agents. The MTT assay however, is one based on the ability of cells to metabolize the MTT into formazan. There is evidence to suggest that some Pluronic co-polymers can cause reduced activity of the electron transport chain (16), which indicates that the results seen are due to an increase of metabolic activity within the mitochondria caused by a non-lethal oxidative stress response. This raises the question as to how long this level of metabolic activity and subsequent oxidative stress response is sustained. And what effect does this stress response have on intracellular pathways such as the NFκB pathway? This pathway is a known regulator of certain pro-inflammatory cytokines such as TNF-α, IL-1β, IL-8, MCP-1/CCL2 and GM-CSF (17). Increased activity of these inflammatory molecules has been shown to play a major role in the activity of matrix metalloproteinases (MMPs), a family of enzymes involved in both tissue degradation and immune responses related to periodontal inflammation.

The clinical relevance is such that the use of some toothpastes containing one of these detergent types may have a detrimental effect on the soft tissues of the gingiva that has not previously been considered, wear by detergents and

toothpastes in general being usually reserved for the hard minerals of the teeth. This wear at the cellular level also has the potential to aggravate inflammation, weaken cell turnover and thus render the mucosa at risk of oral ulceration. It is not known of the protective effects say of saliva in negating the potential damage by detergents.

Again, as with all the studies conducted here, the environment was *in vitro*. No attempt was made to introduce any of the intra oral parameters that may offer protection to the gingiva against chemical agents such as detergents. Indeed it would have been difficult if not impossible to introduce such factors into *in vitro* cell lines, not for the least of which would be microbial contamination. These data nevertheless, if cautiously extrapolated to the oral cavity suggest that some toothpaste detergents could cause damage to the gingival epithelium if not connective tissue.

Of practical relevance it is noted that triclosan which is found in some toothpastes is anti-inflammatory and has been shown to reduce skin irritation challenged by SLS (18). Certainly, detergents such as SLS have considerable anti-microbial activity, being bactericidal at concentrations employed in toothpastes (19). Additionally, in one study using SLS mouth rinses, oral mucosa soreness was reported (20).

There have been a number of studies on the effect of soaps and detergents on the gingival mucosa (9–13). Jacoby *et al.* (13) applied two formulations to the gingiva and concluded that higher concentrations of detergents in toothpastes are likely to break-up the intercellular structure of epithelium and thus provoke increased desquamation.

In conclusion, the two anionic (SLS and Adinol) and one amphoteric (Tego Betain) detergents caused cell death, whereas the non-ionic (Pluronic) detergent may be causing a more complex inflammatory process than simple cell death. It would be prudent to repeat the above experiments with a series of *in vivo* additions such as introducing a 2 min rinse of a detergent solution for volunteers with saliva samples taken before and after, or addition of saliva from individuals with and without periodontal disease to the cells during treatment with detergent solution. Of potential interest would be measuring changes to the content of saliva, particularly relating to presence of molecules related to inflammation, or desquamation of cells from the gingiva.

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