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# Cytokine Release from Organotypic Human Oral Tissue Following Exposure to Oral Care Product Chemicals

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**Purpose:** Measuring viability of a three-dimensional in vitro organotypic human oral tissue model has been suggested as an alternative test method to the oral mucosa irritation test of oral care products. The aim of this study was to investigate the production of two different cytokines using organotypic human oral tissue model following exposure to chemicals that are commonly used in oral care products.

**Materials and Methods:** The organotypic human oral tissues were exposed to ethanol, sodium lauryl sulphate or hydrogen peroxide for 90 minutes. Following exposure, interleukin (IL)-1α and IL-8 productions were assessed and correlated with cell viability testing as well as histology of the organotypic human oral tissues.

**Result:** High levels of IL-8 were released from organotypic human oral tissues in all of the test and control groups without any significant differences between them. In contrast, differences were found in IL-1α release between the test and control groups. Additionally, the trend of IL-1α release corresponded to the phenotypes observed in histological analysis while different trend existed between IL-1α release and cell viability.

**Conclusion:** The study concluded the non-specific release of IL-8 for the assessment of oral care product chemicals' toxicity, while potential of measuring IL-1α cytokine level as the possible alternative test method.

**Key Words:** Cytokines; Dentistry; Safety; Tissues

## Introduction

New oral care products are currently being produced for improved physical, chemical, and biological properties, for which consideration of toxicity of chemicals is necessary for their clinical acceptability[1].

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Both *in vitro* and animal testing have important roles in the evaluation of the biocompatibility of oral care products[2,3]. *In vitro* testing using cell culture techniques has many advantages over animal testing, including a reduced cost, a shorter duration of time required to obtain results, and the ability to screen large numbers of materials[4,5]. However, the clinical relevance of *in vitro* testing is often questioned because of the poor correlation that can sometimes arise between *in vitro* and *in vivo* results. A possible explanation for such discrepancy may be that inflammatory cells are typically not present in *in vitro* systems, which prevents *in vitro* assays from fully reflecting the tissue responses of an animal model[6]. Hence, use of oral mucosa irritation test with animal model has been often considered in many of testing centres, as the method outline in the international standard[7].

With recent propagation of the campaign for animal welfare and the enactment of laws against using animal testing to evaluate cosmetic products/ingredients in the European Union, interests in developing alternative methods of evaluating biocompatibility, with a focus on the 3R principles (reduction, replacement, and refinement), have been rapidly growing[8]. Consequently, attempts have been undertaken to develop *in vitro* biocompatibility testing methods that can simulate and predict biological reactions to dental materials in animal and human models; one example is the three-dimensional organotypic human oral tissue model[9]. Biological studies of numerous and various dental materials such as bonding adhesives[10], orthodontic wires[11], and dental casting alloys[12] have been performed using organotypic human oral tissue models, and several of these studies have produced relevant information that was representative of actual clinical conditions.

Despite that multiple cytokines are involved in the complex animal and human inflammatory reactions that arise following exposure to dental products and materials, many previous studies utilizing organotypic human oral tissue models have only focused on investigating cell viability or considered the release of single cytokines following exposure to dental materials in three-dimensional cell cultures[13,14]. Additionally, a previous study that investigated the release of multiple cytokines from an organotypic human oral tissue model[12] limited the focus only on the biocompatibility of base-metal dental casting alloys.

In consideration of the studies described above, the aim of this study was to investigate the production of two different cytokines using organotypic human oral tissue model following exposure to a collection of chemicals that are commonly used in oral care products: ethanol, which is often used in mouthwash, sodium lauryl sulphate (SLS), which is often used in dentifrices, and hydrogen peroxide ($H_2O_2$), which is used in tooth whitening products. The results from cytokine release assays were then compared with results from both conventional cell viability assays and histological analyses in an attempt to discern the optimal *in vitro* method for evaluating biocompatibility and perhaps to suggest possible alternative test method to animal based oral mucosa irritation test.

## Materials and Methods

### *1. Organotypic Human Oral Tissue Model and Oral Care Product Chemicals*

The organotypic human oral tissue EpiOral™ (MatTek, Ashland, MA, USA) model was used in this study. The model was handled according to manufacturer's instructions and previous studies[14,15]. Briefly, to test to the chemicals of interest, organotypic human oral tissue samples supplied in cell culture inserts were placed into individual wells in standard 6-well plates along with 0.9 ml of culture media. All reagents were provided by the manufacturer. The plates were then incubated for overnight for 16 hours in a 5% $CO_2$ incubator at 37°C.

The samples were exposed to commonly used oral care product chemicals, including 100% ethanol (Sigma-Aldrich, St. Louis, MO, USA), 1% and 3% SLS (Sigma-Aldrich) and 3% $H_2O_2$ (Junsei, Tokyo, Japan). Chemical concentrations were chosen based on what is typical of dental care products and on conditions that were indicated by preliminary experiments to produce significant results. Additionally, 1% Triton X-100 (Sigma-Aldrich) was included as a positive control, and phosphate-buffered saline (PBS, Lonza, Basel, Switzerland) was included as a negative control.

### *2. Cell Viability Following Exposure to Oral Care Product Chemicals*

A 100 µl aliquot of either 100% ethanol, 1% or 3% SLS, or 3% $H_2O_2$ was added to the superficial surface of each organotypic human oral tissue sample for a duration of 90 minutes; the same conditions applied when testing the negative and positive controls. The duration of exposure was determined based on conditions that were used in preliminary experiments that resulted in significant difference between negative and positive control in terms of organotypic human oral tissue viability (results not shown).

Tissue viability was determined for each test and control group using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Sigma-Aldrich), which uses the mitochondrial NADH-dependent cellular oxidoreductase enzyme to generate formazan in an amount that is directly proportional to the number of viable cells[16]. Each sample of treated tissue was washed twice by allowing 10 ml of PBS (Lonza) to overflow into the interior of each cell culture insert. The cell culture inserts were then moved into the wells of a fresh 24-well plate, and 300 µl of MTT that was dissolved in culture media (1 mg/ml) was added per well. An additional 100 µl of MTT solution was then added to the top of each tissue sample, and the plates were incubated for 3 hours in a 5% $CO_2$ incubator at 37°C. Following incubation, the cell culture inserts were again moved into fresh wells in a standard 24-well plate, and 2 ml of isopropanol (Sigma-Aldrich) was added into each insert and well. The plates were shaken for 1 hour, and the cell culture inserts were removed from the wells to enable the optical density (OD) to be measured for each well. OD measurements were taken at 570 nm using a spectrophotometer (Molecular Device, Sunnyvale, CA, USA). Tissue viability was calculated as a percentage based on the OD of the negative control.

### *3. Cytokine Release Assay*

Following the organotypic human oral tissue exposure to the test and control chemicals, as described above, the supernatants from each of the wells were collected. A 50 µl aliquot of each supernatant sample was combined with either 50 µl of mouse anti-human interleukin-1α (IL-1α) or 50 µl of mouse anti-human interleukin-8 (IL-8) (R&D System Inc., Minneapolis, MN, USA) in a standard 96-well plate for 2 hours at room temperature. Additionally, 50 µl aliquots of each of the test and control chemicals were combined with 50 µl aliquots of anti-human IL-1α or IL-8 to confirm that there were no reactions between the chemicals and the antibodies that were used in this study. After the reaction, each well was washed three times with 0.05% Tween 20 in PBS (Sigma-Aldrich), and 50 µl of biotinylated goat anti-human IL-1α or IL-8 (R&D System Inc.) was added to each well. Each well was washed again with 0.05% Tween 20 in PBS (Sigma-Aldrich), and 50 µl of streptavidin-conjugated horseradish peroxidase (R&D System Inc.) was added. After a final wash with 0.05% Tween 20 in PBS (Sigma-Aldrich), the wells were developed with tetramethylbenzidine (Sigma-Aldrich), and the reactions were stopped by the addition of 2 N $H_2SO_4$ (Sigma-Aldrich). The absorbance of each well was measured at 450 nm, and the levels of IL-1α and IL-8 were determined using a calibration curve that was created by measuring absorbance in a series

of dilutions of standard recombinant human IL-1α or IL-8 (R&D System Inc.).

### *4. Histology of Organotypic Human Oral Tissue Samples*

The organotypic human oral tissue samples were exposed to test oral care product chemicals or control materials for 90 minutes as above and fixed with 10% formaldehyde in distilled water (Sigma-Aldrich). They were then paraffin-embedded to be cut into 2 μm sections and stained with haematoxylin and eosin for assessment by histology.

### *5. Statistics*

For statistical analysis, each test was repeated 6 times to obtain the mean and standard deviation values. The t-test was carried out to evaluate the difference between the results obtained for negative control and those obtained from each of the oral care product chemical. Also, difference between the positive control and each of the test sample was assessed with t-test. The SPSS PASW 25.0 program (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Values of $P<0.05$ were statistically significant.

## Result

### *1. Cell Viability Following Exposure to Oral Care Product Chemicals*

The cell viability assay results from the tissue samples that underwent 90-min-long exposures to oral care product chemicals are shown in Fig. 1. Cell viability following exposure to either the positive control or any of the test chemicals was significantly lower ($P<0.05$) than the negative control. There was no difference in cell viability between the positive control (34.6±6.73%) and 1% SLS groups (41.37±5.12%) or between the positive control and 100% ethanol groups (26.2±4.69%). However, the tissues exposed to 3% SLS demonstrated significantly lower cell viability (13.41±0.55%) than the positive control ($P<0.05$). Therefore, 3% SLS was indicated to be the most cytotoxic among the materials assayed. Only exposure to 3% $H_2O_2$ resulted in significantly higher levels of cell viability (51.12±1.03%) than the positive control, suggesting that $H_2O_2$ was the least cytotoxic material assayed.

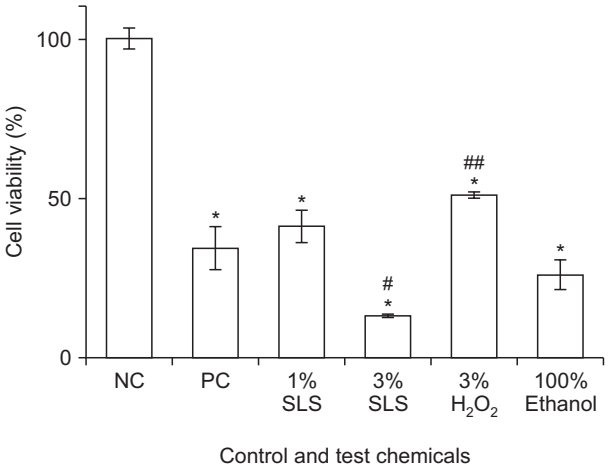


**Fig. 1.** Cell viability of organotypic human oral tissue samples following exposure to control and test chemicals. NC: negative control (phosphate-buffered saline), PC: positive control (1% Triton X-100), SLS: sodium lauryl sulphate, $H_2O_2$: hydrogen peroxide. *Significantly lower than the negative control, #Significantly lower than the positive control, ##Significantly higher than the positive control ($P<0.05$).

### *2. Cytokine Release Following Exposure to Oral Care Product Chemicals*

The results of the cytokine release assays performed on organotypic human oral tissue samples following a 90-min-long exposure to various oral care product chemicals are shown in Fig. 2. The tissue samples released generally high levels of IL-8, regardless of test conditions (Fig. 2A). There was no significant difference in IL-8 release between the positive and negative controls ($P>0.05$). Additionally, there was no significant difference in IL-8 release between the negative control group and any of the test groups ($P>0.05$). Interestingly, 3% SLS that resulted in significantly lower cell viability than positive control showed significantly lower IL-8 release which is opposite to the positive control ($P<0.05$).

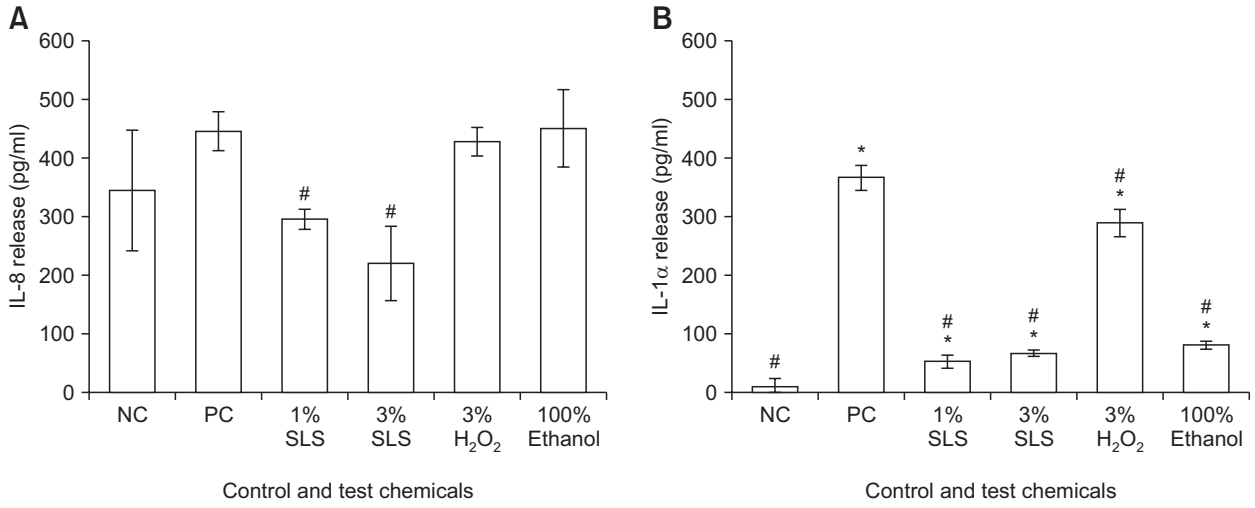


**Fig. 2.** Cytokine release from organotypic human oral tissues following exposure to control and test chemicals. The cytokines that were assays included the following: (A) Interleukin-8 (IL-8) and (B) Interleukin-1α (IL-1α). NC: negative control (phosphate-buffered saline), PC: positive control (1% Triton X-100), SLS: sodium lauryl sulphate, $H_2O_2$: hydrogen peroxide. *Significantly higher than the negative control, #Significantly lower than the positive control (P<0.05).

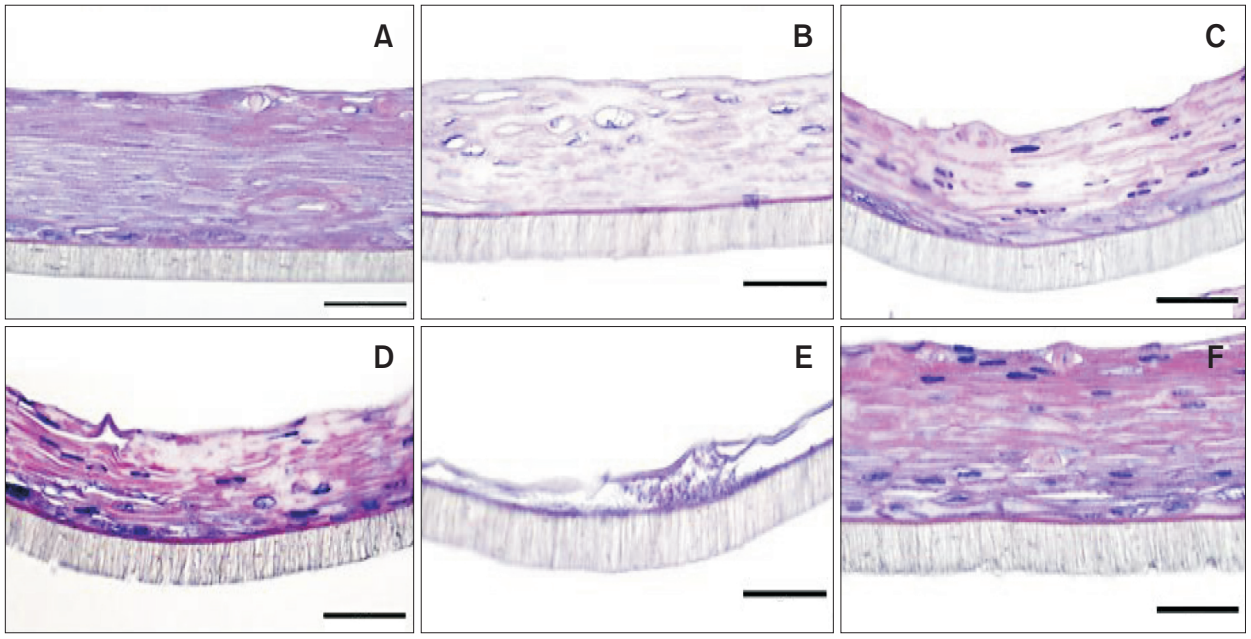


**Fig. 3.** Histology of organotypic human oral tissues following exposure to (A) negative control (phosphate-buffered saline), (B) positive control (1% Triton X-100), (C) 1% sodium lauryl sulphate, (D) 3% sodium lauryl sulphate, (E) 100% ethanol, (F) 3% hydrogen peroxide. The paraffin-embedded tissue was cut into 2 μm sections and stained with hematoxylin and eosin. The scale bar is 30 μm.

In terms of another cytokine examined in this study, there was only a minimal release of IL-1α in tissue samples exposed to the negative control. Conversely, in addition to the positive control, all of the tested chemicals led to significantly higher amounts of IL-1α release than negative control (P<0.05) (Fig. 2B). Additionally, exposure to the positive control resulted in a significantly higher amount of IL-1α release (P<0.05) compared to any of the tested oral care product chemicals (Fig. 2B). Finally, 3% $H_2O_2$ induced the highest levels of IL-1α release compared to any of the other tested chemicals.

### *3. Histology of Organotypic Human Oral Tissue Following Exposure to Oral Care Product Chemicals*

The histological assessments of organotypic human oral tissue samples following a 90 minutes exposure to various oral care product chemicals are shown in Fig. 3. Well-structured, multi-layered, purple-coloured, organotypic human oral tissue was observed following exposure to the negative control. However, a loss of nucleated cells and cell lysis that resulted in pale-coloured tissue were evident with organotypic human oral tissue following exposure to the positive control. With respect to the remaining oral care product chemicals, exposure to 3% $H_2O_2$ was found to cause the most irritation with thin layer of remaining organotypic human oral tissue that was washed away during the process, whereas exposure to 100% ethanol was the least irritating.

## Discussion

Various materials are used as dental care products, and none of them are truly inert in the body[3]. Hence, the evaluation of biocompatibility is an essential step for the research and development of oral care products, although the clinical relevance of the tests has often been questioned[17-19]. As they have many advantages over animal studies, such as reduced cost and reduced time spent screening large numbers of products[4,20], cell culture assays are useful for evaluating the biocompatibility of oral care products. In contrast, animal studies are still considered to be more relevant for biological evaluations and are highly correlated with human clinical models[6].

However, there is growing concern surrounding the welfare of the experimental animals, which has facilitated the enactment of laws that prohibit animal testing of cosmetic product ingredients in the European Union and demands for alternative testing methods[8]. The development of *in vitro* biocompatibility evaluation methods that can serve as alternatives to animal experiments in biochemical, pharmacological and toxicological research has gained widespread popularity in recent years[21]. In this study, we used an organotypic human oral tissue model for *in vitro* biocompatibility evaluation of a selection of oral care product chemicals. In this model, we measured the release of two different cytokines with the goal of investigating a correlation between our results and those produced by conventional methods.

We first performed an MTT assay to assess whether cell viability was associated with cytokine release from organotypic human oral tissue samples after 90 minutes of exposure to oral care product chemicals. All of the tested chemicals led to relatively low measures of cell viability (<50%). SLS is an anionic surfactant that is commonly used as a reference chemical compound when evaluating biocompatibility, as it causes protein denaturation and disrupts phospholipid membranes in cells[22,23]. Additionally, SLS has often been considered to be cytotoxic and is the most widely used agent for cleaning dentifrices or surfactants in dental materials[24-26]; thus, the results generated in this study were not surprising. Ethanol is frequently used as an ingredient in mouthwash to dissolve and preserve essential oils and is well known for producing cytotoxic effects during *in vitro* testing of both two- and three-dimensional cultured cells[15,27]. These findings are in agreement with results that were produced in this study. Our results are also in accordance with those from previous studies that have investigated $H_2O_2$, a commonly used chemical for tooth bleaching. In both cases, low levels of cell viability resulted from exposure to $H_2O_2$[28,29].

Our cytokine release assay produced somewhat different results in the response to different oral care product chemicals. In terms of the two cytokines that were examined in this study, each assay produced a completely different pattern of results. Typically, high levels of IL-8 were released following exposure to any of the control or test chemicals (even the

negative control), and no significant differences were found between the negative and positive controls. The results may be contributed by limitation of the specific organotypic model or by the duration of exposure, which the further studies are currently undergoing to compare the effects on different brands of organotypic human oral tissue model. Still, it could be suggested by results that IL-8 may be unsuitable for use in the evaluation of biocompatibility with the organotypic human oral tissue model and protocols used in this study. Conversely, high levels of IL-1α were detected following exposure to the positive control and low levels were detected following exposure to the negative control. Previous studies have shown that cytokines are constitutively released from organotypic human oral tissue in varying amounts even in the absence of stimulation[30]. Although the exact role and mechanism of cytokine release remains unclear, it has been suggested that IL-1α is primarily produced by oral mucosa keratinocytes, which also regulate the production of additional cytokines, such as IL-8[31]. Hence, following cytotoxic chemical exposure, the sensitivity of our results with respect to IL-1α versus IL-8 may be due to a rapid production of IL-1α in oral tissues and a delay in their regulation of IL-8 production.

Cytokine assay for IL-1α was then compared with cell viability MTT assay results. The results of these assays indicated similar results as a low level of cell viability matched with high level of IL-1α release following the exposure to the positive control, while opposite result for a negative control was evident; a trend that would be expected from relationship between cell viability and cytokine release[12].

However, different results were noted following exposure to several oral care product chemicals that were tested. For example, while 3% $H_2O_2$ resulted in relatively high level of cell viability than other test materials and 3% SLS resulted in the lowest level of cell viability even lower the positive control, 3% SLS released relatively low level IL-1α which was significantly lower than the positive control, and 3% $H_2O_2$ released relatively high level of IL-1α. Additionally, there was no relative difference in cell viability between samples that were exposed to positive control and those that were exposed to other two chemicals; 1% SLS and 100% ethanol, while IL-1α release was significantly lower ($P<0.05$) for two oral care product chemicals compare to positive control.

Finally, the histology of the tissue samples following exposure to control and test chemicals was considered. It was surprising that despite the MTT assay indicated that the level of cell viability for 3% $H_2O_2$ was not significantly lower than positive control, histological results showed thin layer of organotypic human oral tissue following exposure to 3% $H_2O_2$ compare to positive control. This may be due to the nature of $H_2O_2$ that would result in disintegration of cell layer interaction as well as causing cytotoxicity, while other chemicals would simply result in cytotoxicity only[28]. Hence, despite the similar level of cell viability between organotypic human oral tissue exposed to positive control and $H_2O_2$ as indicated by lack of purple coloration for both tissue, cell layer on latter tissue will be easily washed away during the process of histological section preparations. This may indicate the limitation of cell viability assay for organotypic human oral tissue model to replace animal based oral mucosal irritation test, as such *in vitro* assay would fail to consider toxicological effects on multi-layer three-dimensional nature of tissue. Also, despite the similarities in cell viability between positive control and 100% ethanol, it was evident that histological sample exposed to 100% ethanol exhibited much brightly purple coloured specimen than positive control. Finally, 3% SLS that showed significantly lower cell viability even to positive control resulted in relatively thick layer of organotypic human oral tissue model in histology with evidence of bright purple coloured nucleus.

In contrast to dissimilarities between cell viability results and histological results, it was evident that

histological phenotypes were more in agreement with IL-1α cytokine release. First, the highest level of irritation was noted for 3% $H_2O_2$ in both histological model and with IL-1α cytokine release. Also, histological results indicated similarities between 1% SLS, 3% SLS and 100% ethanol, which the level of IL-1α cytokine release was also very similar.

The purpose of the current study was to investigate whether the evaluation of relevant cytokines could serve as an alternative to the cell viability assay, as many current animal tests identify inflammatory reactions that are caused by chemical irritants[7]. Additionally, the release of two different cytokines, IL-1α and IL-8, from organotypic human oral tissue samples was compared to more adequately represent the complex nature of the inflammatory cascades that exist in both animals and humans. The results showed that IL-1α release was better correlated with histology in the organotypic human oral tissue samples that were examined in this study, which was in agreement with previous animal studies and clinical models that tested SLS and ethanol[26,32].

The cell viability is important when evaluating biocompatibility with respect to long-term cell survival[33]. However, the limitation and validity of using MTT-based cell viability assays has often been questioned with regard to the chemicals used and the information they provide in terms of three-dimensional nature of animal or human tissue models[16,34]. Although it is difficult to conclude that assaying IL-1α is a superior method (vs. assaying IL-8 or cell viability) for the evaluation of oral care product biocompatibility when using organotypic human oral tissue models, the results in this study did indicate that this method has potential power. Indeed, the results showed that the release of IL-8 were non-specific in organotypic human oral tissue models for the assessment of oral care product chemicals' toxicity, while IL-1α release was different according to oral care product chemicals and also better correlated with histological findings than cell viability test results. Hence, it may be indicated that there is a possibility of using the levels of IL-1α cytokine released from organotypic human oral tissue samples as the possible alternative test method for the animal based oral mucosa irritation test, though further studies and validations would be required.

## Conclusion

Within the limitation of this study, it was demonstrated that the use of the cytokine level from organotypic human oral tissue samples would be a possible alternative test method for the animal based oral mucosa irritation test. Especially considering the levels of IL-1α cytokine released from organotypic human oral tissue samples in comparison to conventional viability test indicated to be promising. Considering the complicated nature of the inflammatory process and the presence of additional cytokines, further studies that evaluate additional chemicals and cytokines and their correlations with animal or clinical models will be needed along with further development of protocol. Such efforts will help to develop an *in vitro* test method that can serve as an alternative to animal testing.

## Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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