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The use of three-dimensional oral mucosa cell cultures to assess the toxicity of soldered and welded wires

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SUMMARY The aim of the present study was to determine whether there is a difference in toxicity and loss of viability of three-dimensional (3D) reconstructed human oral epithelium (RHOE) cell cultures induced by point-welded (PW), laser-welded (LW), and silver-soldered (SiS) orthodontic wires. Three types of soldered stainless steel (SS) wires: PW, LW, and SiS were prepared (n = 3) and subjected to multiple end-point analysis (MEA). Six pieces were cut from each wire. Each piece was placed on the triplicate cell cultures (RHOE model based on TR 146 cells). After 24 hours of topical exposure, the cell cultures were cut and stained with haematoxylin/eosin. Toxicity was assessed by evaluating the morphological changes and classifying these as mild, moderate, or severe. The *in vitro* cell cultures were subjected to a 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay in order to quantify viability. Copper wires served as the control to determine severe toxicity and native cell cultures were used as a baseline. Untreated SS wire (0.9 mm) was included for comparison with the welded wires.

Histological evaluation with respect to toxicity and measurement of viability in the 3D cell cultures showed no severe toxicity or loss of viability for any of the wires. The morphological ranking of the tested wires from mild to severe toxicity was: SS = PW = LW < SiS. MTT tests revealed the following mean viability values: native cell line (negative control) 98.1 per cent, SS 96.8 per cent, PW 95.5 per cent, LW 95.5 per cent, SiS 85.7 per cent, and copper wires (positive control) 51.2 per cent. Relative comparison between the different welding techniques tested on RHOE revealed, however, that LW and PW wires induced less toxicity/loss of viability compared with SiS wires.

Introduction

In orthodontics, both soldering and welding are standard methods for the application of auxiliaries and for the modification of force systems by joining wires of different cross-sections (Hannemann *et al.*, 1989). The materials used by orthodontists have changed rapidly in recent years and will continue to do so in the future. Disintegration of orthodontic appliances has become a critical issue in orthodontic research because of the potential adverse biological reactions of different materials. Soldered wires, as used in orthodontic appliances, may lead to ionic release and leaching. Oral lesions caused by an orthodontic retainer have been reported (Bishara, 1995). Soldered stainless steel (SS) facebows are very susceptible to corrosion (Grimsdottir *et al.*, 1992b). Large amounts of copper and zinc and some cadmium from the silver solder have been found. Silver-soldered (SiS) SS wire corrodes more than cobalt–chromium, and releases more nickel and chromium than cobalt–chromium wire (Berge *et al.*, 1982). Primarily, as an alternative to soldering, laser welding has been introduced in dental technology (van Benthem and Vahl, 1978; Tambasco *et al.*, 1996). Incomplete filling of the solder gap, porosities resulting from the production process, poor corrosion properties, and, in particular, a high variability leading to insufficient reliability of soldering techniques, confirm the need for alternative joining techniques, such as laser welding (Hofmann and Lindigkeit, 1999; Heidemann *et al.*, 2002).

It is speculated that cytotoxic corrosion products from orthodontic appliances might contribute to localized gingivitis (Grimmsdottir *et al.*, 1992a). In order to limit cytotoxic ion release, investigation of the biological performance of orthodontic materials is important (Eliades *et al.*, 2001). Other studies indicate that metal ions released from metallic biomaterials may have adverse biological effects at concentrations lower than previously reported (Wataha *et al.*, 2000, 2002; Cortizo *et al.*, 2004). In animal studies (Gjerdet *et al.*, 1987), soldered Co-Cr-Ni wire elicited the most severe reactions, and pronounced cytotoxicity was seen. Leachable toxic components of SiS seem to be of importance in the observed tissue response (Gjerdet *et al.*, 1987).

The aim of the present research was to investigate the potential of soldered and welded wires to induce morphological changes in human oral epithelial cell lines and alter the viability as determined by 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay.

Materials and methods

Preparation of the test wires

The orthodontic materials tested in this study were obtained from Forestadent®, Pforzheim, Germany and comprised 0.9 mm SS wire (Forestanit®-wire, spring hard, 0.90 mm 213-6090) and SiS (Silberlot 2.5151 L-Ag72). The composition of the test materials, provided by the manufacturers, is summarized in Table 1. Eight pieces (7 cm in length) were cut, and pairs were united in the middle over a distance of 1 cm by SiS, point welding (PW; Weldman, Bonnel Technology, Schwarzenbruck, Germany) and laser welding (LW; Bego, Dentaurum, Ispringen, Germany). Two pieces of untreated SS wire served as the control. The SiS wire was cleaned and polished, and sterilized before further procedures. From each prepared wire, five 1 mm pieces were cut. This was repeated three times. Copper wire was used as the positive control.

Cell culture

Reconstructed human epidermal and epithelial tissue have been shown to be reliable pre-clinical tools in predicting the irritation potential of topical products (de Brugerolle de Fraissinette *et al.*, 1999; Schaller *et al.*, 2004) and these were used in this study.

The reconstituted human oral epithelial (RHOE) tissue models (SkinEthic Laboratories, Nice, France) consist of airlifted, living, multilayered epithelial tissue constructed of oral epithelium. Cultivated *in vitro* transformed human keratinocytes of the cell line, TR 146 (human cutaneous carcinoma) are produced in polycarbonate inserts in serum-free and chemically defined medium, and form an epithelial tissue (mucosa), devoid of stratum corneum and resembling, histologically, the mucosa of the oral cavity (Rosdy and Clauss, 1990). The inserts containing the RHOE tissue models are shipped at room temperature in a multiwell plate filled with an agarose-nutrient solution in which they are embedded. The multiwell plates are sealed with white tape and packed in a sterile aluminium bag. Maintenance medium, cooled with gel refrigerant, is shipped along with the RHOE human tissue models. The cell cultures of oral human tissue and media are standardized for *in vitro* testing of chemicals or formulations applied on the surface of the tissues or in the medium beneath. Human cells used for the production of the RHOE human tissue models have been tested for biological safety. Absence of HIV integrated proviral DNA, hepatitis C viral DNA, cytomegalovirus DNA, mycoplasma, Hepatitis B antigen HBs, bacteria and fungus are verified by the supplier. The maintenance medium was tested for sterility. However, appropriate safety and sterility precautions were recommended for manipulating human tissue models and media. Each batch of tissues was quality assured by the supplier according to specific quality criteria standards including histology (haematoxylin–eosin staining), tissue viability [MTT mean optical density (OD)] and reproducibility, and tissue thickness (Food and Drug Administration, 1996).

Tissue storage

The 24-well plate was opened under a sterile airflow and the sterile filter paper was removed. The cell culture was placed in a 35 mm Ø culture dish (six-well plate) previously filled with 1000 µl of chemically defined maintenance medium (at room temperature). The RHOE was incubated for 24 hours at 37°C (5 per cent CO_2) before testing. Prior to testing, the medium beneath each 0.5 cm^2 human oral epithelial tissue was changed by adding 500–1000 µl fresh maintenance medium (at room temperature).

Testing procedure

A volume of fresh medium (0.5 ml) was placed under each insert. The different pieces of 1 mm long wire were applied topically onto the surface of the reconstituted epithelial tissues and the culture dishes were placed back in the incubator for 24 hours at 37°C (5 per cent CO_2). Untreated cell cultures served as the negative control.

Histological analysis

For histological analysis, the epithelial tissue, with the polycarbonate filter, was cut off the plastic insert with a sharp scalpel and a standard procedure of paraffin inclusion of the fixed cultures (10 per cent formalin) was performed followed by staining with 0.7/1 per cent haematoxylin/eosin. The results are shown in Figure 1. A scoring system was established by taking into account the overall changes in the morphological parameters. Toxicity was assessed by evaluating the morphological changes and classifying them as mild, moderate, or severe compared with the native

Table 1 Chemical composition of the materials tested, as provided by manufacturers.

Weight %	C	Si	Mn	Cr	Mo	Ni	P	S	Fe	Ag	Cu	Pb	Zn	Sn
Forestanit® 0.9 mm spring hard	<0.12	<1.5	<2.0	16.0–18.0	<0.8	6.0–9.0	<0.045	<0.03	Bal	—	—	—	—	—
Silver solder	<0.005	<0.005	—	—	—	—	<0.005	<0.005	—	71–73	27–29	<0.02	—	—

C, carbon; Si, silicon; Mn, manganese; Cr, chromium; Mo, molybdenum; Ni, nickel; P, phosphorus; S, sulphur; Fe, iron; Ag, silver; Cu, copper; Pb, lead; Zn, zirconium; Sn, tin.

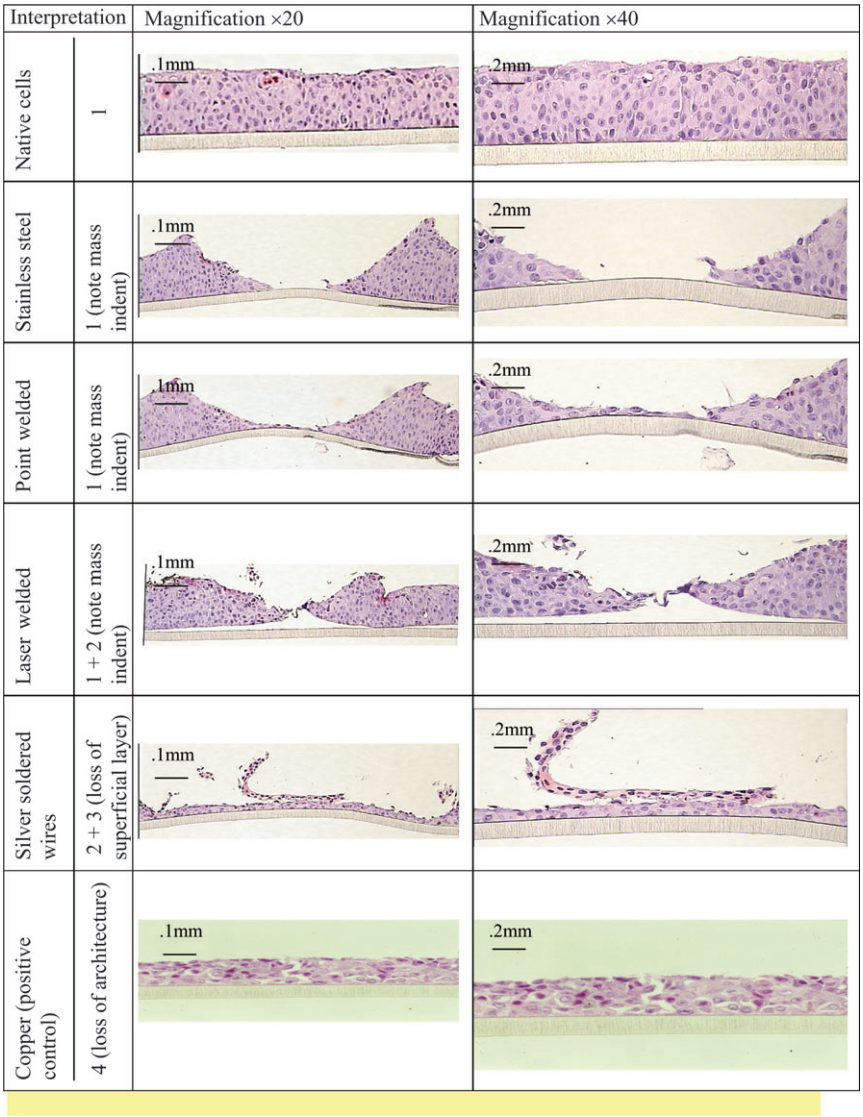

Figure 1 Composite figure of the histological sections.

untreated culture cells (Doucet *et al.*, 1988). For control of the reliability of the histological findings, the results were peer reviewed by two independent observers (anatomo-pathologists).

Quantification of cell viability using the MTT assay

The MTT assay is a simple, accurate and reproducible means for measuring the activity of living cells via mitochrondrial dehydrogenase activity. The key component is MTT. Solutions of MTT, in balanced saline, in the absence of phenol red, are 'yellowish' in colour. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding blue/purple MTT crystals, which are insoluble in aqueous solutions. Crystals formed by viable cells are retained in polycarbonate filters that are used as substratum for the cultures: an intense purple colour of the cultures shows basal cell viability, whereas the colour remains white when necrosis has occurred. Negative control cultures are dark blue, proof of the viability of a cell, and positive control cultures white/yellow as evidence of cell death (Mosmann, 1983; Sjogren *et al.*, 2000, Tomakidi *et al.*, 2000). Quantification of cell viability was performed by calorimetric assay. The MTT crystals were extracted by isopropanol, and the OD of extracts was measured at 570 nm (reference filter 690 nm). The results were expressed as a percentage of viability compared with the negative control. Three hundred microlitres of 0.5 mg/ml MTT (Sigma Aldrich, Bornem, Belgium), in defined culture medium, was added to all test wells and incubated for 3 hours at 37°C (5 per cent CO_2) on

a vibrating platform. After 10 minutes, and 1, and 3 hours, the colour of the wells was observed. After incubation, the MTT solution was discarded. The RHOE was washed twice with 1 ml phosphate-buffered saline pH 7.4 (Life Technologies, Merelbeke, Belgium) at room temperature for 2 minutes, and 1 ml of isopropanol (Merck, Overijse, Belgium) was added to each well. The extraction process was performed over a period of 1 hour at room temperature, on a vibrating platform. Aliquots (200 µl) of blue MTT extraction solution were transferred from each well to the corresponding wells of a 96-well plate. The OD at 570 nm was determined spectrophotometrically using a microplate reader (MIOS, Merck). The mean OD_{570} of the untreated control tissues exposed to culture medium was set to represent 100 per cent of viability, and the results were expressed as a percentage of these controls. The following formula was used:

Percentage of viability = ($OD_{(570–690nm)}$ test product/ $OD_{(570–690nm)}$ negative control) × 100.

Statistical analysis

Statistical evaluation was undertaken with the Statistical Package for Social Sciences (SPSS®, Chicago, Illinois, USA, version 12.0 for Windows) using the Scheffe's method to test all possible contrasts at the same time. Normal distribution of the data was confirmed and the results were checked for homogenicity.

Results

In this study, a multiple end-point analysis (MEA), including tissue viability (MTT conversion) and morphological evaluation by histological interpretation, was performed to determine the cytotoxicity of the tested wires.

Histological interpretation

The slides were evaluated and peer reviewed at a magnification ×20 and ×40 by two histopathologist, who were in agreement (Table 2). The RHOE tissues were sensitive to the majority of the tested wires as there was an indentation at the probe site (Figure 1). The region next to the tested wire was therefore scored. Native cells showed a constant thickness without morphological change. Mild changes were observed for SS, PW, and LW wires. SiS wires showed marked architectural atrophy: most of the upper cell layers were disintegrated but the basal cells remained on the polycarbonate substratum. The ranking of morphological evaluation from mild to severe toxicity was: SS = PW = LW < SiS (Figure 1).

Cell viability with the MTT assay

The qualitative results for the negative control after 10 minutes, and 1 and 3 hours of testing, was blue, indicating that SS, PW, LW, and SiS were non-irritant. The positive control, copper, turned blue/white after 1 hour, evidence of cell death and an acute irritant (Table 3).

The quantitative results were obtained by measuring OD in a calorimeter. To assess the experimental variability, the tested wires were placed in three different wells so that for each wire a mean value could be calculated (Table 4). The mean data ($n = 3$) of the different wires and controls ($n = 6$) which reduced MTT–formazan formation by 50 per cent (MTT_{50}) were, respectively, 0.981 (±0.001) for native cells (negative control), 0.968 (±0.01) for SS, 0.955 (±0.01) for PW, 0.955 (±0.01) for LW, 0.857 (±0.01) for SiS, and 0.512 (±0.01) for copper wire (positive control; Table 3). The percentage of viability is shown in Figure 2. SS, PW, and LW demonstrated normal viability. SiS showed a reduction in viability compared with the native cells and the other tested wires. No acute toxicity of the tested wires was found. Comparison of the MTT values showed, as expected, a statistically significant difference between the mean values

Table 2 Peered assessment of the histological interpretation of toxicity.

Tested material	1	2	3	4
Negative control (−)	✓			
Stainless steel		✓		
Point welded		✓		
Laser welded		✓		
Silver soldered			✓	✓
Copper (+)				✓

(1) Internal negative control cultures: untreated cultures. The epithelial tissues have a constant thickness (corresponding to the control sections), devoid of terminally differentiated cells, and of a regular and compact shape. Cells are attached to each other via multiple desmosomes.
(2) Mild irritant: minimal changes occurring with slight oedema.
(3) Moderate irritant: beginning of a spongious appearance in the upper layers and architectural atrophy, with cellular nuclei irregularity.
(4) Severe irritant (positive control cultures: copper cultures): most of the upper cell layers of the epithelial tissues must be disintegrated, and the remaining basal cells appear loose to the polycarbonate substratum. The presence of spongious, cellular necrosis, loss of cellular junctions in the basal layer, cellular oedema and necrosis in all other cell layers is apparent.

Table 3 MTT_{50} values for repeated tests on different cell cultures.

	10 min	1 h	3 h	Mean value	Standard deviation
Native cells	0.981	0.982	0.979	0.980667	0.001528
Stainless steel	0.968	0.957	0.979	0.968	0.011
Point welding	0.945	0.955	0.965	0.955	0.01
Laser welding	0.966	0.944	0.955	0.955	0.011
Silver solder	0.866	0.846	0.857	0.856333	0.010017
Copper	0.501	0.524	0.512	0.512	0.011504

Table 4 Qualitative evaluation of cell viability.

Tested Product	Colour of cultures			Toxicity
	10 minutes of exposure	1 hour of exposure	3 hours of exposure	
Native (−)	Blue	Blue	Blue	NI
Native (−)	Blue	Blue	Blue	NI
SS T1	Blue	Blue	Blue	NI
SS T2	Blue	Blue	Blue	NI
SS T3	Blue	Blue	Blue	NI
PW T1	Blue	Blue	Blue	NI
PW T2	Blue	Blue	Blue	NI
PW T3	Blue	Blue	Blue	NI
LW T1	Blue	Blue	Blue	NI
LW T2	Blue	Blue	Blue	NI
LW T3	Blue	Blue	Blue	NI
SiS T1	Blue	Blue	Blue	NI
SiS T2	Blue	Blue	Blue	NI
SiS T3	Blue	Blue	Blue	NI
Cu T1	Blue	Blue/white	White	VI
Cu T2	Blue	Blue/white	White	VI
Cu T3	Blue	Blue/white	White	VI

SS, stainless steel; PW, point welded; LW, laser welded; Cu, copper; SiS, silver-soldered wire; T1, T2, T3, repeated test; NI, not irritant; VI, very irritant.

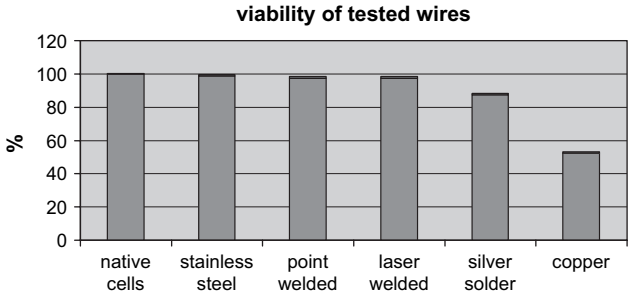

Figure 2 Percentage viability of tested wires (obtained by calorimetry).

of the negative control and all wires examined, and the positive control ($P < 0.05$). SiS resulted in significantly lower viability compared with PW, LW, SS, and native cells, but a significantly higher viability than copper-treated cells ($P < 0.05$; Table 5). The variation between the repeated tests was very low and explains the significant differences (Table 6).

Table 5 Descriptive statistical evaluation, dependent variable MTT (*post hoc* Scheffe test: standard error was 0.008).

Treatment	Mean difference	Significance	95% confidence interval	
			Lower limit	Upper limit
Copper				
Copper				
LW	−0.44264*	0.000	−0.47422	−0.41111
Native cells	−0.46533*	0.000	−0.49989	−0.43678
PW	−0.44267*	0.000	−0.47422	−0.41111
SiS	−0.34400*	0.000	−0.37555	−0.31245
SS	−0.45567*	0.000	−0.48722	−0.42411
Laser welded (LW)				
Copper	0.44267*	0.000	0.41111	0.47422
LW				
Native cells	−0.02567	0.142	−0.05722	0.00589
PW	0.00000	01.000	−0.03155	0.03155
SiS	0.09867*	0.000	0.06711	0.13022
SS	−0.01300	0.752	−0.04455	0.01855
Native cells				
Copper	0.46833*	0.000	−0.43678	0.49989
LW	0.02567	0.142	−0.00589	0.05722
Native cells				
PW	0.02567	0.142	−0.00589	0.05722
SiS	0.12433*	0.000	0.09278	0.15589
SS	0.01267	0.770	−0.01889	0.04422
Point welded (PW)				
Copper	0.44267*	0.000	0.41111	0.47422
LW	0.00000	1.000	−0.03155	0.03155
Native cells	−0.02567	0.142	−0.05722	0.00589
PW				
SiS	0.09867*	0.000	0.06711	0.13022
SS	−0.01300	0.752	−0.04455	0.01855
Silver soldered wire (SiS)				
Copper	0.34400*	0.000	0.31245	0.37555
LW	−0.09876*	0.000	−0.13022	−0.06711
Native cells	−0.12433*	0.000	−0.15589	−0.09278
PW	−0.09867*	0.000	−0.13022	−0.06711
SiS				
SS	−0.11167*	0.000	−0.14322	−0.08011
Stainless steel (SS)				
Copper	0.45567*	0.000	0.42411	0.48722
LW	0.01300	0.752	−0.01855	0.04455
Native cells	−0.01267	0.770	−0.04422	0.01889
PW	0.01300	0.752	−0.01855	0.04455
SiS	0.11167*	0.000	0.08011	0.14322
SS				

Based on observed means. *$P < 0.05$.

Discussion

Compared with native cells, SiS wires showed moderate to severe histopathology with, most markedly, a disintegration of the upper cell layers. The thickness of the cell cultures was not preserved. Since MTT is mainly converted by the basal and suprabasal cells in three-dimensional constructs, to detect necrosis of the upper superficial layer of the tissue model, histological tissue analysis is required (De Wever and Charbonnier, 2002). This confirmed the necessity of a MEA for evaluation of MTT assays. The collected data give a quantification of the cytotoxicity of the tested wires and can explain the adverse effects seen by other authors (Grimmsdottir *et al.*, 1992a; Hofmann and Lindigkeit, 1999; Heidemann *et al.*, 2002). Statistical evaluation demonstrated a significantly lower viability for SiS wires compared with the other tested wires. Gjerdet *et al.* (1987) observed moderate to extreme reactions adjacent to soldered joints and around the wire portion, and found that soldered Co-Cr-Ni wire elicited the most severe reactions caused by leachable toxic components of the silver solder. The results of the present investigation are in agreement with the findings of a

Table 6 Descriptive statistics (dependent variable MTT–Scheffe test).

Treatment	*n**	Subset		
		T1 (10 minutes)	T2 (1 hour)	T3 (3 hours)
Native cells (−)	3			0.98067
Stainless steel	3			0.96800
Point welded	3			0.95500
Laser welded	3			0.95500
Silver soldered	3		0.85633	
Copper (+)	3	0.51233		
Significance†		1.000	1.000	0.142

Means for groups at T1, T2, and T3 in homogeneous subsets are displayed, based on type III sum of squares. The error term is mean square (error) = 0.000.
*Uses harmonic mean sample size = 3.000.
†α = 0.05 (level of significance).

study designed to assess the cell compatibility of orthodontic wires on a fibroblast culture using the MTT test (Rose *et al.*, 1998). Therefore, this RHOE model technique could be used in the future to characterize the effects of dental materials and to reduce the need for animal experiments.

The different histopathology of the SiS and copper wires is in agreement with findings of Wataha *et al.* (1994) who reported that the uptake of Ag^{+1}, Cu^{+2}, and Zn^{+2} was sufficiently 'fast' that *in vivo* exposure of tissues to these metals for periods of less than 12 hours would be capable of disrupting cellular metabolism.

The biocompatibility of a material is not absolute; it must be measured with regard to the way the material is used. Measuring biocompatibility is a complex process that involves *in vitro* and *in vivo* testing. These tests contribute to the understanding of the biological responses to a material but cannot define the biocompatibility of a material with any certainty (Wataha, 2001). The RHOE used in this study recreates many of the structural and functional features of the oral mucosa, but is exclusively composed of keratinocytes. For example, Langerhans cells, which play a critical role in cutaneous immune response and cytokine production, are absent from the RHOE (Srivasta *et al.*, 1994; Cumberbatch *et al.*, 1996). Moreover, the absence of blood-derived and resident leukocytes reduces the complexity of the observable cytokine network. Nevertheless, they are the predominant cell type in human oral mucosa and keratinocytes, and are the first cells in contact with test products, during the initial stages of cellular irritation.

Conclusion

The RHOE tissue model seems to be suitable to demonstrate the relative toxicity of SiS wires. No acute toxicity, however, could be demonstrated. Whether these initial and early responses of the oral keratinocytes are sufficient to detect and discriminate all types of compounds which are irritant to the oral mucosa remains to be determined. This RHOE model technique combined with a MEA could be used in future experiments to test new materials to reduce the need for animal experimentation. Additionally, once validated, this procedure will be less time-consuming and more cost effective than the usual *in vivo* procedures. Further research, however, is needed.

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