Toxicity of used orthodontic archwires assessed by three-dimensional cell culture

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SUMMARY The aim of the present study was to determine whether used orthodontic wires made of different materials cause toxicity and loss of viability on three-dimensional (3D) cell cultures. Three types of orthodontic wires, stainless steel, Nitinol[®], and TMA[®] (n = 9) which had been used clinically in fixed appliances for a period of 1 month, were retrieved at random from five patients. Both upper and lower archwires were collected and subjected to two different protocols: to assess toxicity, two pieces of each wire were placed on 3D cell cultures (reconstituted human epithelium); to investigate the possibility of cell damage, the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay was used and haematoxylin and eosin staining was performed to evaluate morphological changes. Copper wire served as the control to determine the morphology of severe toxicity, and native cell cultures and silk were used as the negative controls.

Morphological evaluation of the native cell cultures revealed no toxic reactions. The ranking, from mild to severe toxicity was as follows: stainless steel < Nitinol® = TMA®. There were no significant differences between TMA® and Nitinol®. The MTT assay revealed the following mean percentage values for viability: native cell line (negative control), 100; stainless steel, 102.25; TMA®, 87.4; Nitinol®, 85.3; and copper wire (positive control) 57.2. Histological evaluation of the 3D cell cultures showed no severe toxicity or loss of viability for any of the wires. However, relative comparison between the different wires revealed that stainless steel induced less toxicity/loss of viability compared with TMA® and Nitinol® wire.

Introduction

It is known that orthodontic materials, such as archwires, may cause adverse clinical effects (Grimsdottir and Hensten-Pettersen, 1993). Clinical use of orthodontic appliances is often associated with an increase in gingivitis, most often related to oral hygiene (Zachrisson and Zachrisson, 1972). Intraoral reactions, such as redness, soreness, and swelling of the oral mucosa, gingiva, and/or lips, have been associated with metal brackets, labial wires, bonding procedures, and acrylic appliances (Jacobson and Hensten-Pettersen, 1989). In some instances, it may be that the appliances or their corrosion products can cause local tissue damage, which cannot clinically be distinguished from gingivitis of a bacteriological aetiology. For a material to be biocompatible, it must exert no toxic effects on the organism. Estimation of cytotoxicity is part of the initial evaluation of biocompatibility (Babich and Sinensky, 2001).

Humans are habitually exposed to a large variety of foreign substances that are potentially toxic and harmful to different organs and tissues. Substances capable of producing cell damage are known as 'toxins' and are classified according to whether they exert their effects in all individuals, in a dose-dependent and predictable manner (intrinsic toxins), or only in some individuals, usually after several contacts, in a non-dose-dependent and therefore unpredictable way (idiosyncratic toxins). Intrinsic toxins may act directly on cellular systems (active toxins) or after biotransformation by hepatocytes (latent toxins). Idiosyncratic toxicity may be the consequence of an unusual metabolism of the drug (metabolic idiosyncrasy) or may be mediated by the immune system after repeated previous contacts (sensitization; Pessayre *et al.*, 1985; Benford and Good, 1987).

Cell culture techniques may be used to assess local cytotoxic effects of solid materials (Schmalz and Schmalz, 1981). Human cell lines have been utilized for a wide variety of purposes in medical research and the number and range available are increasing (Bernard et al., 2002; De Wever and Charbonnier, 2002). Cell culture methods are frequently used to test the biological effects (cytotoxicity) of dental materials, with special emphasis on local irritation (Ames et al., 1973; Hensten-Pettersen, 1981). Cytotoxicity has also been assessed using different methods, e.g. counting surviving cells, measurement of proliferation rates, synthesis of cellular macromolecules, or determination of enzyme activity (Ames et al., 1973). The cytotoxicity of the most common alloys used in orthodontic appliances has been determined by cell culture testing (Locci et al., 2000). It is necessary, however, to consider all the relevant information regarding the derivation of new materials (United States National Bioethics Advisory Commission, 1999).

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Grimsdottir and Hensten-Pettersen (1997), in an optical microscopy study, revealed islands of amorphous precipitants and accumulated microcrystalline particles on used orthodontic wires. They found that after 4 weeks the elemental species precipitated on the material surface were Na, K, and Cl, and after 3 more months Ca and P forming NaCl, KCl, and Ca–P precipitates. Intraoral exposure to orthodontic wires (i.e. NiTi) alters the topography and structure of the alloy surface through surface attack in the form of pitting or crevice corrosion and formation of integuments (Eliades *et al.*, 2000).

To date, all research on cytotoxicity in orthodontics has been carried out on monolayer cell cultures (Rose *et al.*, 1998; Tomakidi *et al.*, 2000). The purpose of the present investigation was to assess the cytotoxic effect of a range of used archwires, in either direct or indirect contact with the oral mucosa during orthodontic treatment, and to quantify this cytotoxic effect and loss of viability by means of a multilayered human epithelial cell culture *in vitro* (Mohebbian, 2001; Mohebbian and Bottenberg, 2003). The development of *in vitro* methods as alternatives to animal experimentation is therefore of relevance in biomedical research aimed at detecting the potential toxicity of xenobiotics in humans (Hensten-Pettersen, 1981).

The quality and specificity of the data generated by *in vitro* models depends on the following factors (Jover *et al.*, 1992; Castell *et al.*, 1997):

- 1. The use of a biological system that reproduces, to a large extent, the metabolic behaviour of the target organ for the toxic effect of the xenobiotic.
- 2. The choice of appropriate parameters for evaluating the toxic effect *in vitro*.
- 3. A correct experimental design so that the *in vitro* data are predictive of the potential *in vivo* effects.

Batch

number

293-512

266-0011

n

3

3

Wire size

0.014 inch

0.017 ×

Product

(manufacturer)

Nitinol® (3M

Unitek, Monrovia, California, USA) TMA® (Ormco-

Materials and methods

The in vitro model: three-dimensional human epithelium cell culture

The three-dimensional (3D) human epithelium model for the in vitro testing of cytotoxicity was supplied by Skinethic[™] Laboratory (Nice, France). The cytotoxic effect was undertaken on containing reconstituted human oral epithelium (TR146 cell line) 0.63-cm² on inert polycarbonate filters. When cultivated at the air-liquid interface in defined medium, the transformed human keratinocytes of the cell line TR146 form an epithelial tissue devoid of stratum corneum, resembling, histologically, the buccal mucosa. The in vitro model and all culture media were prepared without antibiotics and antimycotics. The cell cultures were controlled for biological safety. On a cell pellet of the same strain, the absence of HIV-integrated pro-viral DNA, and hepatitis XC viral DNA was verified as the absence of cytomegalovirus DNA by polymerase chain reaction (Amplicor) and mycoplasma by Hoechst stain. On the supernatant culture, the epithelium was verified for the absence of hepatitis B antigen Hbs and bacteria and fungus. All tests were carried out at the Tissue Culture Laboratories of Skinethic.

Test materials

In order to resemble the clinical situation as closely as possible, three types of orthodontic wires (Table 1), used in fixed appliances and potentially xenobiotic, were collected from five patients [three females and two males, with an age range of 12–15 years (average 13.2 years)] treated in a private orthodontic practice. The orthodontic appliances consisted of four bands and 20 bonded brackets. All wires (n = 9) were collected at random from the upper and lower arch after an intraoral exposure period of 1 month. Two 1 mm sections of the wires were cut 5 mm from the distal

Zr

6

Sn

4.5

Mo

11.5

Fe

Cr

С

0.03

0.08

 Table 1
 Used orthodontic wires and metal composition of different components in orthodontic wires expressed in percentage of weight and rounded to the nearest integer (as provided by the manufacturers).

Weight percentage

Si

Ni

54

Ti

46

78

SDS, Orange, California, USA)			0.025 inch									
Stainless steel (S30400; GAC International, Bohemia, New York, USA)	03-622-63	3	0.016 × 0.022 inch	2	1	8	_	_	_	_	69	19

Mn

Mn, manganese; Si, silicon; Ni, nickel; Ti, titanium; Sn, tin; Zr, zirconium; Mo, molybdenum; Fe, iron; Cr, chromium; C, carbon.

end and each piece was disinfected with alcohol (Nedalco-Des-O 380, Belgaco NV, Gent, Belgium) before testing.

Testing procedure

A multiple end-point analysis was performed. A sample of the used wire to be tested was deposited directly on the reconstituted mucosa. Native cells and silk were used as internal negative controls and copper wire as the positive control (Schmalz et al., 1997). Triplicate cultures were incubated at 37°C, 5 per cent CO_2 for 24 hours. The viability of the keratinocytes of the basal cell layer was evaluated on duplicate cultures and the effect of the tested product on the cultures' histology verified on the third culture. For each condition, at the end of the test period, the treated cultures were rinsed twice with phosphate-buffered saline solution, and placed in 300 µl of 0.5 mg/ml 3-(4,5dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT; Mosmann, 1983). Bioassays which monitor changes in metabolic activity or decreases in cell number are generally accepted methods to evaluate the adverse effects of xenobiotics on cells (Schmalz, 1982). Included in this group of bioassays is the MTT test (Mosmann, 1983; Tomakidi et al., 2000).

The MTT assay is designed to be used for non-radioactive spectrophotometric quantification of cell proliferation and viability in cell populations using the 96-well plate format (Sjogren *et al.*, 2000). This assay is based on cleavage of the tetrazolium salt MTT, in the presence of an electron-coupling reagent, by active mitochondria. The water-insoluble formazan salt produced has to be solubilized in an additional step. After this incubation period, a water-insoluble formazan dye is formed. After solubilization, the formazan dye is quantitated using a scanning multiwell spectrophotometer: the MTT crystals are extracted by isopropanol and the optical density (OD) of extracts is measured at 570 nm (reference filter 690 nm). The results are expressed as a percentage of viability compared with the negative control.

For qualitative evaluation of cell viability, the colour of each culture was noted after 10 minutes and 1, 3, and 24 hours: negative control cultures have to be a dark blue colour, proof of the cell's viability [non-irritant (NI)], and positive control cultures blue/white or white, evidence of cell death [very irritant (VI)].

For quantitative evaluation of cell viability after an incubation period of 3 hours in 300 μ l of 0.5 mg/ml MTT, the cultures were placed in 2 ml of isopropanol. Extraction was performed at 37°C, for a minimum of 1.5 hours, by gentle shaking. OD was measured on 200 μ l of extract at 570 nm (reference filter 690 nm). The percentage of viability values was calculated as the OD reading of the probe divided by the OD reading of the negative control multiplied by 100. The results are thus expressed as a percentage of viability compared with the negative control [mean \pm the standard

deviation (SD) of duplicate cultures]. The following formula applies:

$\begin{aligned} \text{Percentage of viability} &= \text{OD}_{(570-690\,\text{nm})} \text{ test product} \\ \text{OD}_{(570-690\,\text{nm})} \text{ negative control} \times 100. \end{aligned}$

In order to verify the results of the MTT assay, histology is mandatory. The MTT assay is active on all cells without taking into account possible necrosis of the upper cell layers. As the *in vitro* model comprises different cell layers, MTT assay results have to be controlled (De Wever and Charbonnier, 2002).

For each of the tested orthodontic wires or controls, at the end of each test period, the cell cultures were cut in half. The two treated cultures were fixed in a balanced 10 per cent formalin solution and later embedded in paraffin. Vertical sections (4 μ m) were stained with haematoxylin and eosin, and photographed under a light microscope. The colour photomicrographs of the stained sections were compared with untreated native cell cultures.

For histopathological interpretation, a scoring system (Doucet *et al.*, 1988) was established by taking into account the overall changes in the morphological parameters (Figure 1).

For control of the reliability of the histological findings, the results were peered by two independent observers (anatomo-pathologists)

Results

The different cross-sections of the used orthodontic archwires were evaluated for their capacity to induce cytopathic effects on human buccal mucosa reconstituted by *in vitro* multilayered cell culture of transformed keratinocytes. The results of the qualitative evaluation of cell viability are shown in Table 2. Negative control cultures, as well as stainless steel and silk, were blue and thus were NI. TMA® and Nitinol® were blue/white after 24 hours and were labelled as slightly irritant. Positive controls were blue/white or white, evidence of cell death and VI.

The SD of the different readings (n = 6) in the colourimeter for the MTT assay was calculated but showed little difference. The MTT test revealed the following mean percentage values for viability: native cells (negative control), 100; silk, 95.32; stainless steel, 102.25; TMA®, 87.4; Nitinol®, 85.3; and copper wire (positive control), 57.22.

No acute toxicity was found between the tested wires. The eccentricity of the difference was highly significant (P < 0.05), not only between the copper (positive control) and stainless steel wire but also between Nitinol® and TMA® versus stainless steel (Tables 3 and 4).

The peered morphological histological findings, using the histological index (Figure 1), revealed no toxic reactions. The ranking of the morphological evaluation from mild to severe toxicity was stainless steel < Nitinol < TMA. When compared with the negative and positive controls, only mild changes were observed between the tested wires.

Table 2Qualitative evaluation of cell viability: negative control cultures have to be a dark blue colour, proof of the cell's viability[non-irritant (NI)], and positive control cultures blue/white [very slight irritant (VSI)] or white, evidence of cell death [very irritant (VI)].Products tested undiluted.

Tested Product	Colour of cultures					
	10 minutes of exposure	1 hour of exposure	3 hours of exposure	24 hours of exposure		
Native cells (-)	Blue	Blue	Blue	Blue	NI	
Native cells (-)	Blue	Blue	Blue	Blue	NI	
Silk	Blue	Blue	Blue	Blue	NI	
Silk	Blue	Blue	Blue	Blue	NI	
Nitinol®	Blue	Blue	Blue	Blue/white	VSI	
Nitinol®	Blue	Blue	Blue	Blue/white	VSI	
TMA®	Blue	Blue	Blue	Blue/white	VSI	
TMA®	Blue	Blue	Blue	Blue/white	VSI	
Stainless steel	Blue	Blue	Blue	Blue	NI	
Stainless steel	Blue	Blue	Blue	Blue	NI	
Copper (+)	Blue	Blue/white	White	White	VI	
Copper (+)	Blue	Blue/white	White	White	VI	

Table 3 Quantitative cell viability: results of optical densitometry at 570 nm after 24 hours incubation at 37°C with MTT assay.

Tested materials	First reading	Second reading	Third reading	Mean value	Calculated mean (SD)
Native cells (-)	0.341	0.361	0.3620	0.354667	0.3845 (0.058)
Native cells (-)	0.396	0.423	0.424	0.414333	
Silk	0.352	0.373	0.374	0.366333	0.3665 (0.015)
Silk	0.353	0.374	0.373	0.366667	
Nitinol®	0.317	0.334	0.333	0.328000	0.328 (0.0106)
Nitinol®	0.316	0.336	0.332	0.328000	× /
TMA®	0.325	0.355	0.350	0.343333	0.336167 (0.004)
TMA®	0.312	0.344	0.331	0.329000	
Stainless steel	0.417	0.403	0.424	0.414667	0.393167 (0.034)
Stainless steel	0.364	0.383	0.368	0.371667	
Copper (+)	0.182	0.201	0.199	0.194000	0.22 (0.05)
Copper (+)	0.235	0.249	0.254	0.246000	

MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide; SD, standard deviation.

The cell cultures for stainless steel showed the most preserved architecture. For TMA® and Nitinol®, there was only slight modification in the architecture (Figure 2a,b,c), although the appearance of apoptosis was observed (Figure 3).

The results of the MTT assay are presented diagrammatically in Figure 4.

Discussion

The ultimate advantage of the 3D culture system is to allow direct topical application of insoluble materials onto a mucosal surface in order to reproduce, as closely as possible, the topical application achieved *in vivo*. Most of the metals used in the oral cavity can be expected to undergo some type of corrosion (Grosgogeat *et al.*, 2003; Oh *et al.*, 2004; Yonekura *et al.*, 2004). Although nickel is not highly toxic, small amounts can cause allergic reactions (Greppi *et al.*, 1991; Bishara *et al.*, 1993; Grimsdottir *et al.*, 1994; De Silva and Doherty, 2000). Compared with stainless steel, TMA® and Nitinol® showed more toxicity when assessed on *in*

vitro mucosa. The specific metal responsible for the toxicity was not determined. In the histopathological analysis of TMA®, Nitinol®, and stainless steel, some cells showed the appearance of apoptosis with, at the light microscopic level, single dispersed cells with condensed eosinophilic cytoplasm and, at an early stage, masses around the margins of nuclei, or the entire nucleus appeared deeply basophilic (Figure 3). Apoptosis is a genetically controlled, metabolically active, evolutionarily conserved process by which cells self-destruct (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Steller, 1995; Thompson, 1995; Cummings *et al.*, 1997). This is in agreement with the findings of David and Lobner (2004) who reported that stainless steel induced apoptosis.

The used orthodontic wires were obtained at random from five different patients and were not preserved in standard saliva. This means that the used wires, taken crosssectionally, were not standardized or under the same conditions. Different external attacks, such as food intake (acids, sugar, etc.), as well as temperature and mechanical factors, cannot therefore be evaluated. MTT and histology



Figure 1 Histological classification index (light microscopy ×40) of *in vitro* oral epithelium. (a) The epithelial tissues have a constant thickness, devoid of terminally differentiated cells, with a regular and compact shape. Cells are attached to others via multiple desmosomes. (b) Minimal changes occur with slight oedema. (c) The beginning of spongious tissue development in the upper layers, with architectural atrophy, and cellular irregularity. (d) Most of the upper cell layers of the epithelial tissues become disintegrated, and the remaining basal cells show loose adhering to the polycarbonate substratum. There is spongious tissue development, cellular necrosis, and loss of cellular junctions in the basic layer together with cellular oedema and necrosis in all other cell layers. Some cells appear to show apoptotic bodies.

 Table 4
 Quantitative cell viability of tested materials.

	Percentage viability (OD probe/ OD neg × 100)	SD percentage	Calculated mean value	SD
Native cells (-)	100.00	10.97	0.384500	0.35001
Silk	95.32	0.06	0.366500	0.10858
Nitinol®	85.31	0.00	0.328000	0.009011
TMA®	87.43	2.64	0.336167	0.009011
Stainless steel	102.25	7.91	0371667	0.016388
Copper(+)	57.22	9.56	0.22000	0.029893

OD, optical density; neg, negative; sd, standard deviation.



Figure 2 Histology results of tested wires (light microscopy ×40) assessed with classification index (Table 5). (a) Nitinol® (b + c): mild and moderate tissue disruption. (b) TMA® (b + c): mild and moderate tissue disruption. (c) Stainless steel (a + b): normal to mild tissue disruption.

were analysed on control (negative and positive) as well as on the different used wires (n = 9), such that potential inhomogenicity in one group can be reduced to an acceptable level using this *in vitro* model. The MTT assay revealed 102.25 per cent values for viability for stainless steel
 Table 5
 Peered assessment of the results of the histological interpretation using a histological index.

Tested material	А	В	С	D
Native cells (-)	1			
Silk	\checkmark	1		
Nitinol®		\checkmark	\checkmark	
TMA®		\checkmark	\checkmark	
Stainless steel	1	\checkmark		
Copper (+)				1

(a) The epithelial tissues have a constant thickness, devoid of terminally differentiated cells, with a regular and compact shape. Cells are attached to others via multiple desmosomes.

(b) Minimal changes occur with slight oedema.

(c) The beginning of spongious tissue development in the upper layers, architectural atrophy, and cellular irregularity.

(d) Most of the upper cell layers of the epithelial tissues become disintegrated, and the remaining basal cells demonstrate loose adherence to the polycarbonate substratum. There is spongious tissue development, cellular necrosis, and loss of cellular junctions in the basal layer together with cellular oedema and necrosis in all other cell layers. Some cells appear to show apoptotic bodies.



Figure 3 Histological paraffin section of reconstituted human oral epithelium stained with haematoxylin and eosin showing the appearance of apoptotic bodies (magnification $\times 200$).



Figure 4 Viability of tested materials with 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide assay.

archwires compared with untreated tissues (MTT values of 100 per cent). This phenomenon is described as 'hormesis' and is a stress response in cells exposed to low levels of heavy metals (Damelin *et al.*, 2000)

Conclusion

While clinical conditions can never deliver standardized laboratory pre-conditions, laboratory conditions can never simulate the full range of different influences in the oral cavity. Therefore, both clinical and laboratory investigations are necessary.

Histological evaluation showed no severe toxicity or loss of viability caused by any of the tested orthodontic wires; nevertheless relative differences were found.

Histopathological analysis of the positive control and the used Nitinol® and TMA® wires showed necrosis and apoptosis. The multiple end-point analysis with comparison between the different used wires revealed that stainless steel induced less toxicity/loss of viability compared with Nitinol® and TMA®. This suggests that stainless steel wires should be used as soon as biomechanically possible.

None of the examined used orthodontic wires were found to exhibit acute cytotoxicity, regardless of the type of test employed.

The human reconstituted epithelium model provides a constant, stable, quantifiable, and reproducible method for further in-depth *in* vitro studies of orthodontic materials.

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