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In vitro toxicity evaluation of silver soldering, electrical resistance, and laser welding of orthodontic wires

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SUMMARY The long-term effects of orthodontic appliances in the oral environment and the subsequent leaching of metals are relatively unknown. A method for determining the effects of various types of soldering and welding, both of which in turn could lead to leaching of metal ions, on the growth of osteoblasts, fibroblasts, and oral keratinocytes *in vitro*, is proposed. The effects of cell behaviour of metal wires on osteoblast differentiation, expressed by alkaline phosphatase (ALP) activity; on fibroblast proliferation, assayed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenil)-2H-tetrazolium–phenazine ethosulphate method; and on keratinocyte viability and migration on the wires, observed by scanning electron microscopy (SEM), were tested.

Two types of commercially available wires normally used for orthodontic appliances, with a similar chemical composition (iron, carbon, silicon, chromium, molybdenum, phosphorus, sulphur, vanadium, and nitrogen) but differing in nickel and manganese content, were examined, as well as the joints obtained by electrical resistance welding, traditional soldering, and laser welding. Nickel and chromium, known as possible toxic metals, were also examined using pure nickel- and chromium-plated titanium wires. Segments of each wire, cut into different lengths, were added to each well in which the cells were grown to confluence.

The high nickel and chromium content of orthodontic wires damaged both osteoblasts and fibroblasts, but did not affect keratinocytes. Chromium strongly affected fibroblast growth. The joint produced by electrical resistance welding was well tolerated by both osteoblasts and fibroblasts, whereas traditional soldering caused a significant ($P < 0.05$) decrease in both osteoblast ALP activity and fibroblast viability, and prevented the growth of keratinocytes *in vitro*. Laser welding was the only joining process well tolerated by all tested cells.

Introduction

Orthodontic appliances are increasingly used in young and adult subjects; clinical practice, however, has shown that the long-term presence of metals in the oral environment can be harmful to health. All materials currently available have European authorization and general safety certification which, however, do not prevent the possibility of phenomena caused by their long-term presence in the mouth or individual sensitivity. Mucosal ulcerations, caused by orthodontic appliances, severe irritations, allergies to nickel etc., especially associated with soldered joints have been reported (Kvam *et al.*, 1989; Bishara, 1995; Marigo *et al*., 2003; Rahilly and Price, 2003; Kalimo *et al.*, 2004; Saglam *et al.*, 2004; Schultz *et al.*, 2004). A number of studies have found satisfactory results in which laser welding is used to join titanium prostheses to implants (Wang and Welsch, 1995; Knabe and Hoffmeister, 2003).

In view of these clinical observations, the aim of this study was to investigate the effects of different types of welding materials on the proliferation of cells in the oral cavity. Previous investigations dealing with the biocompatibility of orthodontic wires utilized animal cells (Grimsdottir *et al.*, 1992; Mockers *et al.*, 2002) or human gingival fibroblasts (Locci *et al.*, 2000; Eliades *et al.*, 2004), while the present research was concerned with human osteoblasts, fibroblasts, and keratinocytes.

A preliminary study (data not shown) considered the effects of different metallic wires and silver soldering or welding on osteoblast proliferation *in vitro*. Osteoblasts showed a decrease in alkaline phosphatase (ALP) activity, which is considered a marker of cell differentiation and viability. This decrease increased with increasing nickel content of the wire. The aim of the present research was to extend the study to relatively pure metals (nickel and chromium, the latter tested with chromium-plated titanium wires, since titanium is considered inert), and then to traditional silver soldering and both electrical resistance and laser welding. Even if osteoblasts are not in direct

contact with orthodontic materials in the oral cavity, it cannot be excluded that they may be affected. The effects of metallic materials and welds were also tested on normal human skin fibroblasts, used as a model for connective tissue cells, and oral mucosal keratinocytes, developing and using different *in vitro* tests.

Materials and methods

The compositions of the two commercially available orthodontic wires, the chromium-plated titanium wire and the soldering alloy tested, as provided by the manufacturers, are reported in Table 1. The first two wires differed from each other essentially in manganese (16–20 to 2 per cent, respectively), molybdenum (1.8–2.5 to less than 0.8 per cent, respectively), and nickel (less than 3 to 6–9 per cent, respectively). Chromium was plated on a titanium 5 grade (6 per cent aluminium, 4 per cent vanadium) wire. The diameter of all the wires was 1 mm.

The wires underwent traditional silver soldering, electrical resistance, or laser welding. The latter was carried out by Leone SpA (Florence, Italy), who manufactured and supplied all the orthodontic materials.

Tests on osteoblasts

SaOS-2 cell lines were cultured in six-well plates with Coon's medium (Imperial, Unipath Milan, Italy) and 10 per cent foetal calf serum (FCS), in a 5 per cent CO_2 atmosphere at 37°C. At confluence, this was replaced with steady-state medium (0.5 per cent FCS) for 48 hours. Six segments of each wire, which were cut into different lengths (from 15 to 30 mm) to cover the majority of the cells, were added to each well, in triplicate for each sample. After 48 hours, the medium was removed, the cells washed twice in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.5), and lysed with 0.1 percent Nonidet (Nonylphenyl-polyethylene glycol, Nonidet P-40 substitute, Fluka-Sigma-Aldrich Co., St Louis, Missouri, USA). The lysate was centrifuged at 2000 *g* for 10 minutes and the ALP activity assayed using the Sigma procedure 104. (This is based on the hydrolysis of *p*-nitrophenyl phosphate, yielding inorganic phosphate and *p*-nitrophenol, which is converted to a yellow complex readily measured at 400–420 nm. The intensity of colour formed is proportional to the phosphatase activity.) Protein content was assayed in the supernatant using the method of Bradford (1976; this was partially modified after demonstrating Nonidet interference, not described by the author). This method for protein determination involves the binding of Coomassie brilliant blue G-250 to protein, causing an increase in absorption at 595 nm.

To evaluate the effect of the sole compression of the added materials, osteoblast cultures were first tested using porcelain discs (Vitadur Alpha A3, Vita Zahnfabrik, Bad Sakingen, Germany) which is considered an inert material (Sjögren *et al.*, 2000).

Tests on fibroblasts

Primary cultures of human skin fibroblasts obtained from healthy volunteers were performed according to Carneiro Coelho and Giugliani (2000). They were cultured with Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich Co.) supplemented with 10 per cent FCS, in a 5 per cent CO_2 atmosphere at 37°C. A total of 1200 cells were inoculated into every well of a 96-well plate in the presence of a single wire of each sample, 5 mm length (8 wells for each sample). In order to determine the number of viable cells in proliferation, the MTS–PES colorimetric method was applied, using CellTiter 96®AQu$_{eous}$ One Solution Reagent (Promega Co., Madison, Wisconsin, USA). This reagent contains a novel tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)

Table 1 Composition (%) of the tested wires and soldering material.

	Wire 1	Wire 2	Nickel	Chromium	Soldering
Carbon	<1.0	<0.12	0.2		
Silicon	<1.0	Max 1.5	0.11		
Manganese	16–20	Max 2	0.27		
Chromium	16–20	16		Chromium-plated*	
Molybdenum	1.8–2.5	<0.8			
Nickel	<3.0	6.0–9.0	99.56		
Phosphorous	<0.05	<0.045			
Sulphur	0.05	<0.03			
Vanadium	<0.2				
Nitrogen	0.7–1.0				
Copper			0.02		22
Iron	Residue	Residue	0.02		
Silver					56
Zinc					17
Tin					5

*Titanium 5 grade alloy (6% aluminium, 4% vanadium) was plated with chromium.

-2-(4-sulphophenil)-2H-tetrazolium, inner salt, MTS, and an electron coupling reagent: phenazine ethosulphate, PES. MTS is bioreduced by cells into a coloured formazan product; this reaction is accomplished by NAD(P)H produced by dehydrogenase enzymes in metabolically active cells. At cell confluence, 20 µl of CellTiter 96®AQu$_{eous}$ One Solution Reagent were added to each well, the plate was incubated for 2 hours at 37°C, and the absorbance recorded at 490 nm with a 96-well plate reader (Versa$_{max}$ tunable microplate reader, Molecular Devices, Sunnyvale, California, USA). DMEM with wires, but without cells, was used as the control.

Tests on keratinocytes

Primary keratinocyte cultures were obtained from oral mucous membrane fragments, as previously described (Pianigiani *et al.*, 1999). The cells were then seeded onto human de-epidermized dermis (DED) containing the wires to be tested and inserted in an oblique position with respect to the cell layer. The cell suspension and the wires were retained on the DED with a stainless steel ring. The cultures were prepared for scanning electron microscopy (SEM) 14 days later. The specimens were fixed with 2.5 per cent glutaraldehyde, 5 per cent paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, rinsed with 0.1 sodium cacodylate buffer, post-fixed in 1 per cent osmium tetroxide, dehydrated through a graded ethanol series, and dried in a CO_2 critical point dryer. Dried samples were mounted with conductive cement on stubs, sputter coated with 10 nm gold/palladium, and viewed with a Philips SEM 505 (Philips Netherland B.V. Eindhoven, The Netherlands).

All the experiments were repeated at least three times. The data for osteoblast ALP and fibroblast CellTiter assays are the mean values ± the standard error, expressed as a percentage of non-treated cells. The statistical analysis was performed using analysis of variance with a $P \leq 0.05$ as the limit of statistical significance. Each reported image of keratinocytes is one of four replicate specimens showing the same result. Osteoblast and fibroblast growth was monitored daily by light microscopic observation (TMS inverted microscope, Nikon Co., Tokyo, Japan).

Results

Tests on osteoblasts

No difference in ALP activity was found between osteoblast cultures with or without porcelain discs.

As shown in Figure 1, taking the activity of non-treated cells as 100 per cent, the preliminary observations (unpublished data) on the effect of orthodontic wires with different nickel contents on osteoblast viability were confirmed. ALP activity detected in the cells grown in the presence of untreated wires was 71.9 ± 6.9 and 75.8 ± 5.6 per cent for wires 1 and 2, respectively, both showing a statistically significant difference with regard to the controls ($P < 0.05$). With pure nickel wires, the cells showed lower ALP activity (54.7 ± 10.1 per cent) than with the commercial wires. Chromium inhibited ALP activity of the osteoblasts grown in its presence to 31.3 ± 4.4 per cent ($P < 0.05$).

The wires welded by electrical resistance resulted in a decrease in cell ALP activity to 54.6 ± 22.2 (wire 1) and 51.8 ± 11.3 (wire 2) per cent, with cell viability approximately 40 per cent. The decrease of activity caused by wire 2 was statistically significant with respect to the controls ($P < 0.05$).

Osteoblasts grown in the presence of silver-soldered wires showed a dramatic decrease in ALP activity compared with the corresponding but non-soldered wires, reaching 49.4 ± 13.2 (wire 1) and 40.5 ± 5.7 (wire 2) per cent of the control values. The difference with respect to the controls was statistically significant ($P < 0.05$). Light microscopic

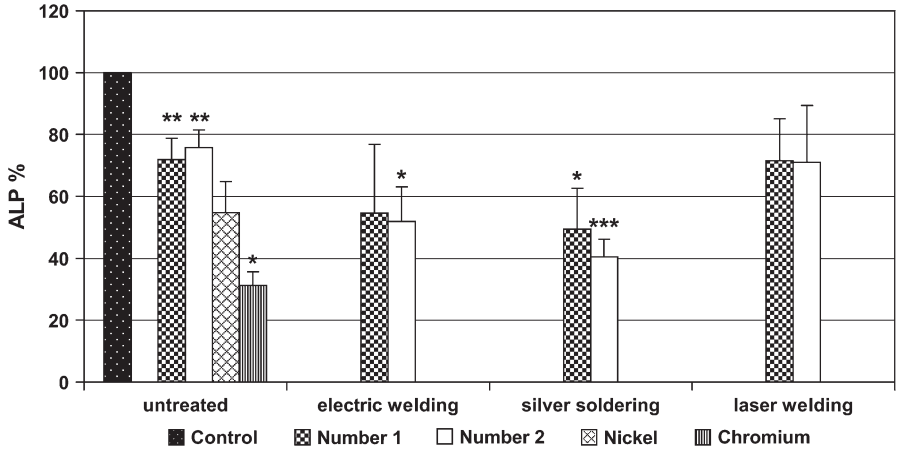

Figure 1 Alkaline phosphatase activity of osteoblasts expressed as a percentage with respect to the controls. Cells were grown in the presence of wires 1 and 2, and nickel- and chromium-plated wires. The wires were untreated or soldered by electrical resistance, silver soldering, or laser welding. All data are means ± standard error of 10 experiments, analysed by analysis of variance. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$.

observation showed clear areas of necrosis near welds, so that only the more distant cells survived. Cell viability, assayed by the Trypan blue exclusion test, was approximately 11 per cent.

No difference in either ALP activity (wire 1: 71.5 ± 13.5 and wire 2: 70.9 ± 18.3 per cent of control values) was observed between the cells grown in the presence of laser soldered or control wires.

Tests on fibroblasts

Preliminary tests were performed to determine the relationship between fibroblast number and absorbance at 490 nm in the presence of CellTiter 96®AQu$_{eous}$ One Solution Reagent. These data then allowed a calculation of the number of viable cells in proliferation in each well. The numbers of cells in each well containing metallic specimens were compared with the number of non-treated cells, taken as 100 per cent.

The number of cells grown in the presence of wire 1 was 46.4 ± 8.16 per cent with respect to the controls, showing a statistically significant difference ($P < 0.05$; Figure 2), while the number grown in the presence of wire 2 was 77.1 ± 11.3 per cent and those grown with pure nickel wires 63.0 ± 24.4 per cent. As observed for osteoblasts, chromium was the most toxic of the tested metals; the proliferation of fibroblasts was entirely inhibited.

Electrical welding did not affect cell growth. At the end of the experiment, fibroblasts growth in the presence of both welded wires was 62.1 ± 18.51 and 89.7 ± 20.7 per cent of controls, respectively. Fibroblasts grown in the presence of the silver-soldered wires were less than the controls, reaching 10.1 ± 11.8 and 8.3 ± 17.5 per cent of control values, respectively. The difference was statistically significant ($P < 0.05$). The number of the cells grown in the presence of laser welded wires 1 and 2 reached 45.6 ± 17.1 and 49.1 ± 22.1 per cent of the controls, respectively.

Tests on keratinocytes

Keratinocytes grew well on both wires, showing regular stratification and differentiation during culture. After 14 days of culture, actively proliferating cells were observed, which migrated over the wire inserted into DED, colonizing it and re-establishing a complete epithelium (Figure 3a). Thus, to test the capacity of these cells to proliferate on welds, only wire 1 was used.

The cells stopped migrating at the traditional soldered joints (Figure 3b), but grew and stratified well on laser welds (Figure 3c).

Discussion

The orthodontic wires tested were not particularly toxic for osteoblasts, fibroblasts, or keratinocytes in culture. Osteoblast ALP activity slightly declined for both wires, but was statistically significant. Fibroblast growth was inhibited by wire 1 but not by wire 2, while keratinocyte proliferation was not affected by either wire.

Nickel is the most common metal causing allergy in predisposed patients (Rahilly and Price, 2003; Kalimo *et al.*, 2004; Saglam *et al.*, 2004; Schultz *et al.*, 2004) but no evidence of its direct toxicity has yet been clearly demonstrated (David and Lobner 2004; Eliades *et al.*, 2004). The results of the present study show that nickel caused a slight depression of cell activity, but without statistical significance. Chromium-plated titanium wire caused a sharp decrease in ALP activity and fibroblast death. This resulted in severe toxicity to the cells probably due to the high chromium ion concentration released by the layer—the plating being quite unstable. All these observations suggest that the slight negative influence of wires 1 and 2 on osteoblasts and fibroblasts is due to the presence of chromium (16–20 per cent) in the alloy, even if there is limited release by stainless steel, more than to nickel presence.

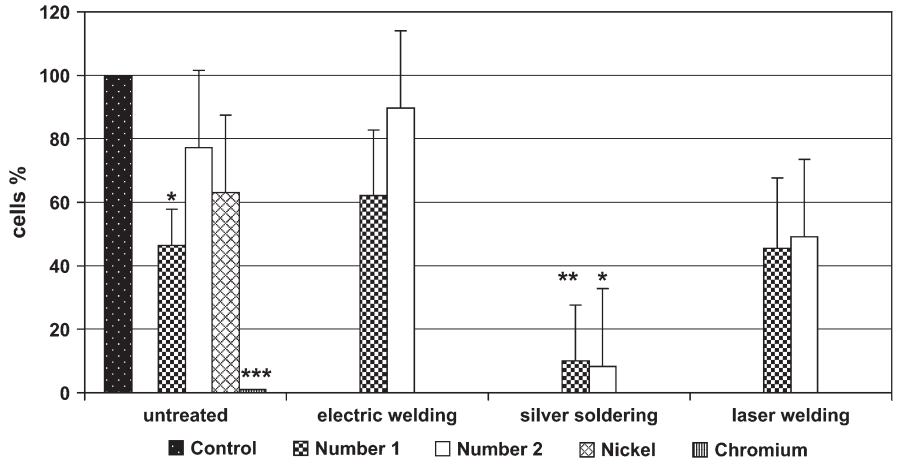

Figure 2 Number of viable fibroblasts in proliferation, expressed as a percentage with respect to the controls. Cells were grown in the presence of wires 1 and 2, and nickel- and chromium-plated wires. The wires were untreated or soldered by electrical resistance, silver soldering, or laser welding. All data are means ± standard error of five experiments, analysed by analysis of variance. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$.

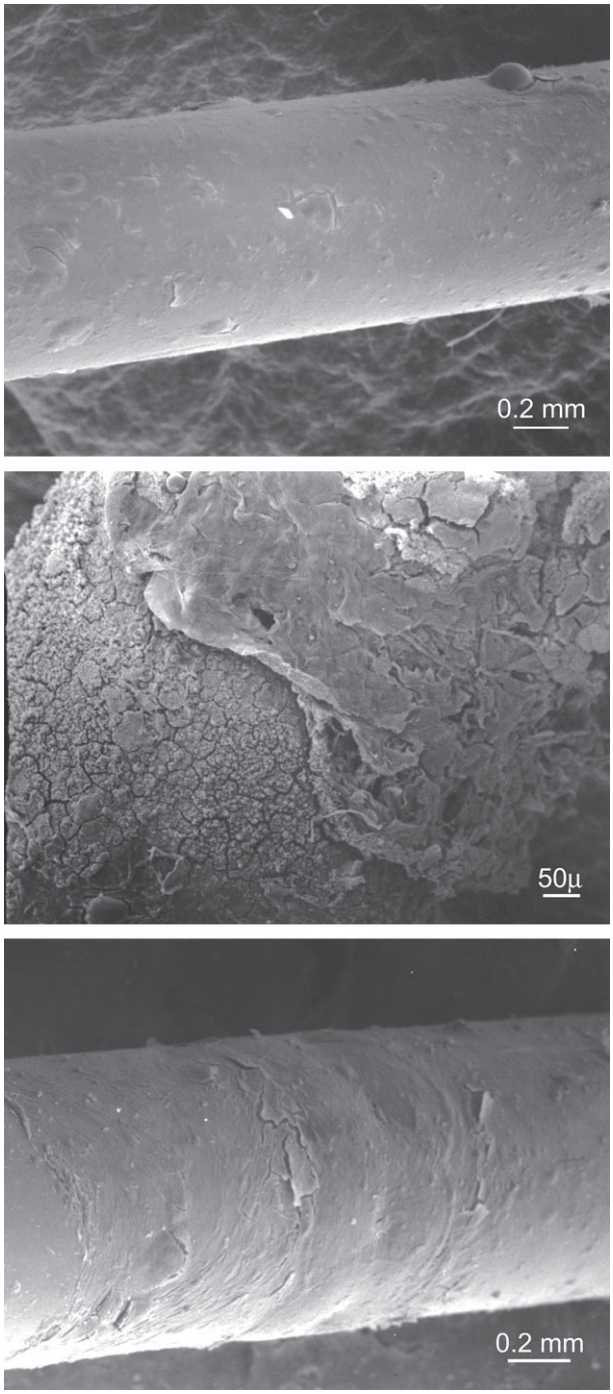

Figure 3 Keratinocytes migrating over untreated (a), silver soldered (b), and laser welded (c) wire.

Silver soldering was highly toxic for all types of cells considered in this study. It caused a sharp decrease in osteoblast ALP activity, indicating the end of the differentiation process, and the death of the cells proliferating in its environment. It also caused severe depression of fibroblasts proliferation and was highly toxic for keratinocytes, which failed to migrate over the welds, while proliferating well on the wire itself. The cytotoxicity of silver solder has been shown in mouse fibroblasts, caused by molar bands joined with silver- and copper-based brazing alloys, but not in single-component devices (Grimsdottir *et al.*, 1992). Studies on mouse fibroblasts have demonstrated the cytotoxicity of only one, clinically used, out of 10 tested molar bands, leading the authors to suggest a concomitant action of the presence of soldering and clinical use (Mockers *et al.*, 2002).

Two possible reasons for weld toxicity could be surface roughness, which prevents keratinocytes from adhering to the wire, and/or the release of metal ions by the weld, preventing growth of osteoblasts and fibroblasts in its vicinity. These observations suggest that physico-chemical modifications produced during soldering are the main cause of cell damage. A previous report concerning the adhesion of monocytes to titanium implant surfaces (Soskolne *et al.*, 2002) showed that cell adherence increases with increasing surface roughness. In the absence of similar direct evidence for the cell types tested in the present research, and considering the different and particular functions of monocytes, it is hypothesized that there is a concomitant role of chemical and physical modifications in the toxic action of soldering. Further experiments are nevertheless needed to clarify this.

The present evidence of the inhibition of keratinocyte migration over welds is also significant for orthodontics. The presence of welds in the mouth can interfere with the healing of mucosal wounds by inhibiting keratinocyte migration in the last stages of the healing process.

Laser welds were the only ones well tolerated by cells, especially keratinocytes, which due to their direct contact with them are more affected by orthodontic devices.

Conclusions

The findings show that the different commercially available orthodontic wires investigated are not particularly toxic for the cells. Electrical resistance welding is well tolerated, while traditional silver soldering is toxic for osteoblast differentiation, fibroblast viability, and keratinocyte growth. Laser welding was well tolerated by all cells tested, thus showing its high biocompatibility.

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