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In vitro cytotoxicity of orthodontic archwires in cortical cell cultures

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SUMMARY There have been a number of studies regarding the toxicity of orthodontic archwires, but little is known concerning the mechanism of their toxicity. This investigation used murine cortical cell cultures to examine the *in vitro* neurotoxicity of commonly used orthodontic metallic archwire alloys. The materials examined included 0.016 inch nickel-titanium (NiTi), copper-nickel-titanium, titanium-molybdenum, Elgiloy, and stainless steel archwire alloys. Standard sized samples of each material were placed on tissue culture inserts suspended above the cell cultures. Neuronal death was determined using the lactate dehydrogenase release assay 24 hours after exposure to the archwires.

The results indicated that NiTi, copper-nickel-titanium and titanium-molybdenum alloys were not neurotoxic, while stainless steel and Elgiloy were significantly toxic. Washing the archwires for 7 days in a saline solution did not alter the toxicity. However, the free radical scavenger, trolox, blocked the toxicity of both stainless steel and Elgiloy, indicating that the death was free radical mediated. The caspase inhibitor, Z-VAl-Ala-Asp-fluoromethylketone (zVAD-FMK), blocked the toxicity of stainless steel, but not Elgiloy, suggesting that stainless steel induced apoptosis. Further evidence that stainless steel induced apoptosis was provided by propidium staining which showed nuclear chromatin condensation and fragmentation into discrete spherical or irregular shapes, characteristic of apoptosis. The specific metal responsible for the toxicity was not determined; the metals common to each of the toxic archwires were nickel, iron, and chromium.

Introduction

A number of characteristics have been used to describe the properties of an ideal orthodontic archwire. The wire should have a large springback, low stiffness, good formability, high stored energy, biocompatibility, environmental stability, low surface friction, and the capability to be welded or soldered to auxiliaries (Kapila and Sachdeva, 1989). Currently no single orthodontic wire alloy has all of these ideal qualities and each wire is therefore selected on the basis of its strengths and limitations.

The biocompatibility of archwires is largely determined by their metal composition. The presence of nickel in some archwires has been of particular concern. Nickel allergy is the most common contact allergy in females in the USA and Europe (Staerkjaer and Menné, 1990; Bass *et al.*, 1993). Allergic reactions to orthodontic appliances have been reported in the literature (Dunlap *et al.*, 1989). In a study of both corrosion and cellular immune responses to nickel-containing archwires, it was found that the quantity of nickel released from wires in synthetic saliva was 700 times lower than the concentration of metal required to produce cytotoxic effects in human peripheral blood mononuclear cells from both nickel-sensitive and nickel-non-sensitive individuals (Jia *et al.*, 1999). Nickel and chromium levels in the blood of patients prior to orthodontic treatment, and 2 and 5 months following the start of treatment, revealed that corrosion from these appliances did not increase blood levels of these two metals even in circumstances where nickel-titanium (NiTi) wires were used during treatment (Bishara *et al.*, 1993).

Metals other than nickel are present in orthodontic archwires. The potential for toxicity due to the release of each of these metals exists. The aim of this investigation was to study the *in vitro* neurotoxic effects of commonly used metallic orthodontic archwires on murine cortical cells. This study represents the first known characterization of the effects of orthodontic materials on cortical cell cultures. The most commonly used orthodontic archwire alloys, NiTi, copper-nickel-titanium, titanium-molybdenum, Elgiloy, and stainless steel, were investigated. Further experiments were conducted to characterize the mechanism of toxicity for those wires that caused significant toxicity.

Materials and methods

Materials

Timed, pregnant, Swiss Webster mice were obtained from Charles River Laboratories (Wilmington, Delaware, USA). The following were investigated: NiTi, copper-nickel-titanium, titanium-molybdenum (0.016 inch diameter) archwires (Ormco/Sybron, Glendora, California, USA),

stainless steel (0.016 inch diameter) archwires and Transbond™ XT light cure adhesive (3M Unitek, Monrovia, California, USA), Blue Elgiloy (0.016 inch diameter) archwires (Rocky Mountain Orthodontics, Denver, Colorado, USA), and Band-Lok™ adhesive (Reliance Orthodontic Products Inc., Itasca, Illinois, USA). The exact composition of each archwire is given in Table 1. The archwires were cut into standard sized pieces (average weight = 2.9 ± 0.1 mg). Five pieces of each wire were then placed on tissue culture inserts suspended above the cultured cells. In this way the elastics were in contact with the media bathing the cultures, but not in direct contact with the cells. This system avoids possible cell damage due to physical disruption of the cells. Serum was obtained from Life Technologies (Gaithersburg, Maryland, USA) and Z-VAl-Ala-Asp-fluoromethylketone (zVAD-FMK) from Research Biochemicals International (Natick, Massachusetts, USA). All other chemicals were obtained from Sigma (St. Louis, Missouri, USA).

Cortical cultures

Mixed cerebral cortical cell cultures containing both neuronal and glial cells were prepared from foetal (15–16 day gestation) mice as previously described (Asrari and Lobner, 2001). Dissociated cortical cells were plated on 24-well plates coated with poly-D-lysine and laminin in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5 per cent heat-inactivated horse serum, 5 per cent foetal bovine serum, 2 mM glutamine and glucose (total 21 mM). Cultures were maintained in humidified 5 per cent CO_2 incubators at 37°C. The mice were handled in accordance with a protocol approved by the institutional animal care committee.

Table 1 Composition of the tested orthodontic archwires*.

Category of material	Composition (%)
NiTi	54 nickel 46 titanium
CuNiTi	49.10 nickel 45.64 titanium 5.00 copper 0.20 chromium 0.06 carbon
TMA	~ 80–85 titanium 10–13 molybdenum 3.75–5.25 tin 0.10 carbon
Elgiloy®	39 cobalt 21 chromium 16 iron 14 nickel 8 molybdenum
Stainless steel (Unitek)	72 iron 18 chromium 8 nickel 1 manganese

*According to the most current company material safety data sheets.
NiTi, nickel-titanium; CuNiTi, copper-nickel-titanium; TMA, titanium-molybdenum alloy.

Lactate dehydrogenase (LDH) release assay

Cell death was quantitatively assessed by the measurement of LDH, released from damaged or destroyed cells, in the extracellular fluid 24 hours after the insult was started. Blank LDH levels were subtracted from insult LDH values, and the results normalized to 100 per cent neuronal death caused by addition of the glutamate receptor agonist, 500 μM N-methyl-D-aspartate (NMDA). Control experiments have previously shown that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of neurons damaged or destroyed (Koh and Choi, 1987; Gwag *et al.*, 1995).

Propidium iodide staining

The cultures were exposed to the non-vital dye propidium iodide (10 μM) for 30 minutes, following 24 hours of exposure to the archwires. Digital images were taken with a Diagnostic Instruments spot camera at a magnification of ×400 and presented as black and white images.

Statistical analysis

Statistical calculations were performed using one-way ANOVA followed by Dunnett's *post hoc* test. $P < 0.05$ was considered to indicate significant differences.

Results

The *in vitro* neurotoxicity of orthodontic metallic archwires was determined using cortical cell cultures. The alloys tested included NiTi, copper-nickel-titanium, titanium-molybdenum, Elgiloy, and stainless steel. After a 24 hour exposure, only Elgiloy and stainless steel caused significant neurotoxicity (Figure 1). The cultures used for these experiments contained both neuronal and glial cells. However, Elgiloy and stainless steel appeared to cause the death of only neurons, as similar exposure of the alloys to pure glial cultures did not cause any toxicity (data not shown).

Experiments were conducted to characterize the effects of washing the toxic alloy samples and to determine the mechanism of their toxicity. Washing the Elgiloy and stainless steel samples with Eagles' MEM for 7 days did not significantly affect the toxicity compared with unwashed samples (Figure 2). The effects of a free radical scavenger, trolox, an NMDA-type

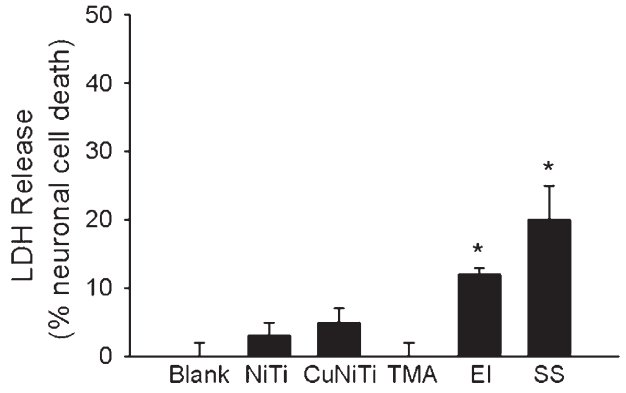

Figure 1 Toxicity of archwires in cortical cell cultures. The bars show percentage cell death (mean ± standard error of the mean, n = 8–16). Cell death was quantified by measuring the release of lactate dehydrogenase (LDH) 24 hours after the beginning of the insult. Sham wash values were subtracted, and the results were scaled to the level measured in sister cultures exposed to 500 μM N-methyl-D-aspartate (NMDA) for 24 hours (= 100, this exposure induced near-complete neuronal death). *Significant toxicity. NiTi, nickel-titanium; CuNiTi, copper-nickel-titanium; TMA, titanium-molybdenum alloy; El, Elgiloy; SS, stainless steel.

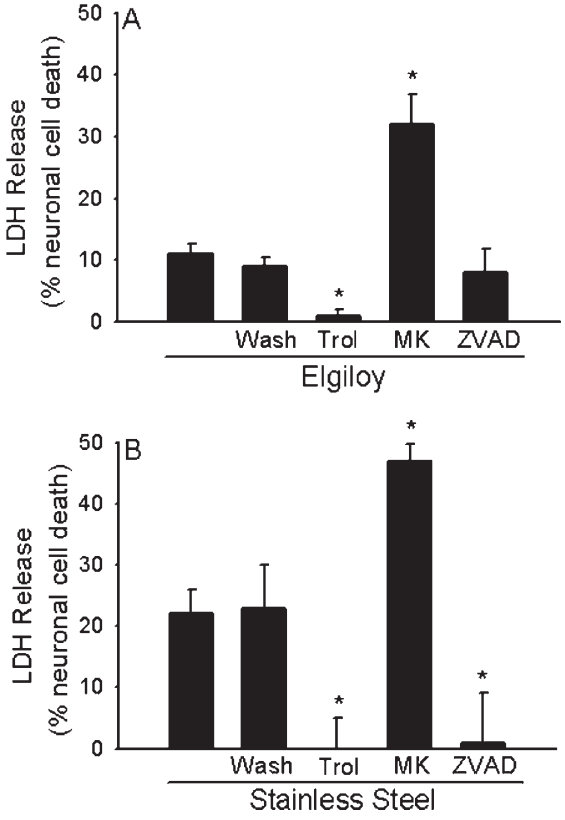

Figure 2 The effects of washing and potential protective agents on Elgiloy (A) and stainless steel (B) toxicity. The bars show percentage cell death (mean ± standard error of the mean, n = 8–16). *Significant difference from control toxicity (alloy without any drug). Wash, alloys washed in culture media for 7 days; Trol, 100 μM trolox (free radical scavenger); MK, 10 μM MK-801 [N-methyl-D-aspartate (NMDA) receptor antagonist]; ZVAD, 100 μM Z-VAl-Ala-Asp-fluoromethylketone (zVAD-FMK; caspase inhibitor).

glutamate receptor antagonist, MK-801, and a caspase inhibitor, zVAD-FMK, were also tested on the toxicity induced by Elgiloy and stainless steel (Figure 2). Trolox significantly attenuated the neurotoxicity induced by Elgiloy and stainless steel. MK-801 did not attenuate, and in fact potentiated, the toxicity of both Elgiloy and stainless steel. zVAD-FMK was protective against stainless steel toxicity, but not Elgiloy toxicity. This last result suggests that stainless steel induced apoptosis, while Elgiloy induced necrosis.

The type of death induced was further investigated using propidium iodide staining. In control cultures there was very little propidium iodide staining (Figure 3a), indicating that most cells were alive. Exposure to the protein kinase inhibitor, staurosporine, which is known to induce apoptosis in this culture system (Koh *et al.*, 1995), caused nuclear chromatin condensation and fragmentation into discrete spherical or irregular shapes characteristic of apoptosis (Figure 3b). Exposure to NMDA, which is known to induce necrosis in this culture system (Gwag *et al.*, 1997), caused diffuse nuclear staining indicating generalized, random, DNA breakdown, characteristic of necrosis (Figure 3c). Exposure to Elgiloy (Figure 3d) caused nuclear staining similar to NMDA, while stainless steel (Figure 3e) resulted in staining similar to that caused by staurosporine. The propidium iodide staining induced by stainless steel was largely eliminated by the addition of zVAD-FMK (Figure 3f).

Discussion

Previous studies of the *in vitro* toxicity of archwires have been carried out using fibroblasts (Ryhänen *et al.*, 1997; Wever *et al.*, 1997; Rose *et al.*, 1998; Es-Souni *et al.*, 2003), osteoblasts (Ryhänen *et al.*, 1997; Wever *et al.*, 1997), and smooth muscle cells (Shih *et al.*, 2000, 2001). The culture system used in the current investigation was a primary neuronal culture. The main difference between this system and those previously used is that neurons are highly susceptible to many insults (Mattson *et al.*, 1995). However, the results are generally consistent with previous studies in that Elgiloy and stainless steel were toxic (Rose *et al.*, 1998). Research regarding the toxicity of NiTi archwires has been variable, with indications that it is both toxic (Shih *et al.*, 2000, 2001) and non-toxic (Ryhänen *et al.*, 1997; Wever *et al.*, 1997). The present results indicated no toxicity of NiTi archwires.

The lack of protective effect provided by the NMDA-type glutamate receptor antagonist MK-801 indicates that the release of glutamate was not a factor in the toxicity of the archwires. The potentiation of the injury by MK-801 was somewhat surprising. The addition of MK-801 attenuates a number of different insults in this culture system (Monyer *et al.*, 1989; Koh and Choi, 1994), although a blockade of glutamate receptors for

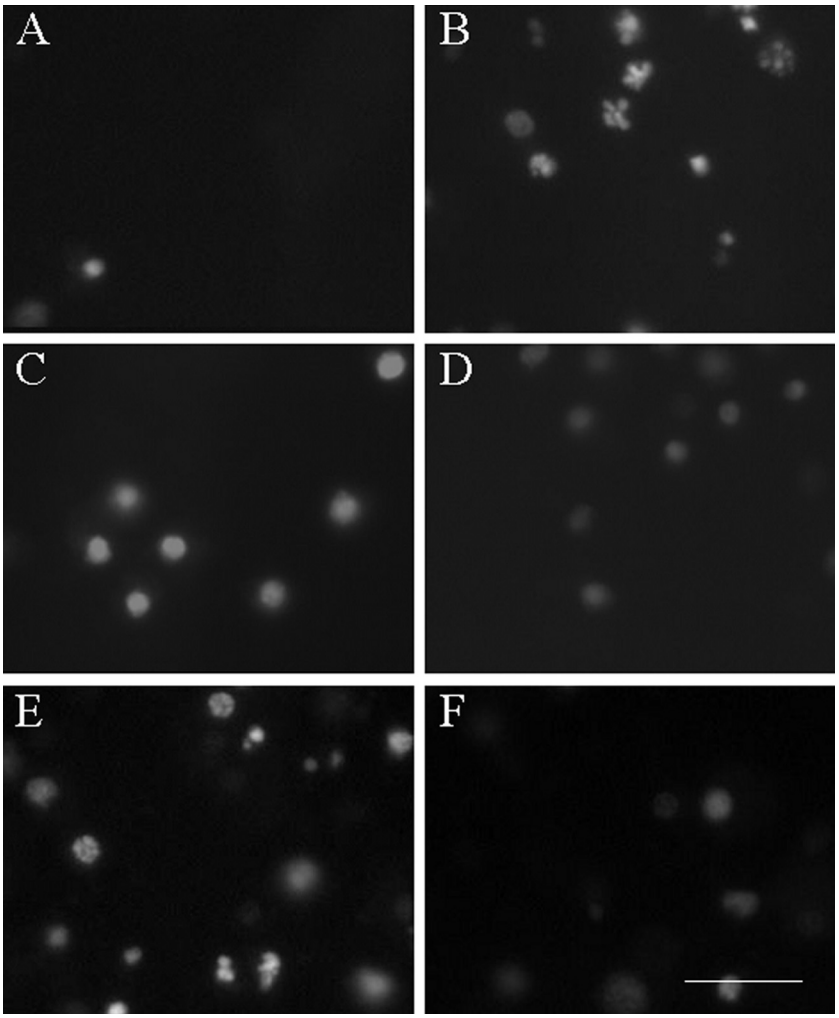

Figure 3 Propidium iodide staining following sham wash (A), 200 μM staurosporine (B), 10 μM N-methyl-D-aspartate (NMDA) (C), Elgiloy (D), stainless steel (E), or stainless steel plus 100 μM Z-VAl-Ala-Asp-fluoromethylketone (zVAD-FMK) (F). Propidium iodide (10 μM) was added for 30 minutes, 24 hours after the beginning of the insult. Bar = 50 μm.

an extended period of time can cause toxicity (Hwang *et al.*, 1999).

It was not definitively determined what metal was responsible for the toxicity of Elgiloy and stainless steel. However, given the composition of the toxic and non-toxic archwires (Table 1), it appears likely that the toxic component was nickel, iron, or chromium. Nickel is also present at much higher levels in NiTi and copper-nickel-titanium archwires, which were not toxic. However, these archwires also contain titanium which is known to decrease the release of metals due to passive film formation (Gil *et al.*, 1999). The fact that the toxicity was blocked by the free radical scavenger trolox also argues for nickel, iron, or chromium being the toxic component. Nickel, iron, and chromium can each enhance free radical production through a Fenton-like reaction (Torreilles *et al.*, 1990; Halliwell and Gutteridge, 1992; Sugden *et al.*, 1992) and each is known to be neurotoxic (Diaz-Mayans *et al.*, 1986; Evan *et al.*, 1995; Lobner *et al.*, 2003).

The type of cell death, apoptosis or necrosis, caused by Elgiloy and stainless steel was determined. Apoptosis, or programmed cell death, involves a fundamental series of morphological changes (Kerr *et al.*, 1972). Apoptosis plays an important role in the physiological turnover of normal cells and the maintenance of tissue homeostasis. Moreover, it can be observed in cells following exposure to specific toxins (Granchi *et al.*, 2000). The other mechanism by which eukaryotic cells die is necrosis. This degenerative process occurs in response to severe hypoxia, lytic viral infections, complement attack, hyperthermia, and exposure to toxic chemicals (Kerr *et al.*, 1972; Granchi *et al.*, 2000). Many apoptotic pathways depend on the activation of caspases or cysteine proteases that act to cleave cytosolic and nuclear proteins, ultimately leading to apoptosis (Kidd, 1998). The caspase inhibitor, zVAD-FMK, acts to block the activation of these enzymes and is known to block apoptosis in this culture system (Lobner, 2000).

Consequently, the decreased level of cell death observed for stainless steel-exposed cortical cultures in the presence of zVAD-FMK suggests death by apoptosis. Propidium iodide staining of the archwire-exposed cultures was used to identify morphological differences between apoptotic and necrotic cells (Lobner *et al.*, 2003). Cortical cultures exposed to Elgiloy exhibited diffuse nuclear staining typical of necrosis, while cultures exposed to stainless steel demonstrated chromatin condensation and fragmentation into irregular shapes, features characteristic of apoptosis. Taken together, the pharmacological results using zVAD-FMK and the morphological results obtained with propidium iodide staining, it is clear that Elgiloy induces necrosis and stainless steel induces apoptosis.

Iron causes necrosis in cortical cell cultures (Lobner *et al.*, 2003). There are no data concerning the type of cell death caused by nickel or chromium in cortical cell cultures. However, nickel and chromium are known to induce apoptosis in other cell types (Chen *et al.*, 2001; Kim *et al.*, 2002). Because stainless steel induced apoptosis and Elgiloy induced necrosis, the results are most consistent with iron being responsible for Elgiloy toxicity, with nickel or chromium being responsible for stainless steel toxicity. However, measurement of the release of the metals will be required to definitely determine the metal responsible for the toxicity of the archwires. It should also be noted that no attempt was made to mimic clinical conditions which would involve the presence of factors such as ageing of the archwires and pH fluctuations of the bathing media.

It appears unlikely that the release of any metal from orthodontic archwires presents a health risk. Nickel, iron, and chromium are ubiquitous in the diet and ingestion of high levels is required to cause health problems (Anderson, 1997; Schumann, 2001). The fact that these alloys release metals at sufficiently high levels to cause neurotoxicity is not of great concern regarding archwires, but may be a consideration in situations where these alloys are used in restricted spaces where the released metals could accumulate.

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