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Cytotoxicity and genotoxicity of orthodontic bands with or without silver soldered joints

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ABSTRACT

Stainless steel bands, with or without silver soldered joints, are routinely used in orthodontics. However, little is known about the toxic biological effects of these appliances. The aims of this study were to evaluate the cytotoxic, cytostatic, genotoxic and DNA damage-inducing effects of non-soldered bands (NSB) and silver soldered bands (SSB) on the HepG2 and HOK cell lines and to quantify the amount of ions released by the bands. The 24-h metallic eluates of NSBs and SSBs were quantified by atomic absorption spectrophotometry. An MTT reduction assay was performed to evaluate the cytotoxicity, alkaline and modified comet assays were employed to measure genotoxicity and oxidative DNA damage effects, and cytokinesis-block micronucleus cytome (CBMN-Cyt) assays were used to verify DNA damage, cytostasis and cytotoxicity. Ag, Cd, Cr, Cu and Zn were detected in SSB medium samples, and Fe and Ni were detected in both the SSB and NSB medium samples. The SSB group induced stronger cytotoxic effects than the NSB group in both evaluated cell lines. NSB and SSB induced genotoxicity as evaluated by comet assays; stronger effects were observed in the SSB group. Both groups induced similar increases in the number of oxidative DNA lesions, as detected by the FPG and Endo III enzymes. Nucleoplasmic bridges, biomarkers of DNA misrepair and/or telomere end fusions, were significantly elevated in the SSB group. The SSB eluates showed higher amounts of Ni and Fe than NSB, and all the quantified ions were detected in SSB eluates, including Cd. The SSB eluates were more cytotoxic and genotoxic than the NSB samples. Based on these results, we propose that other brands, materials and techniques should be further investigated for the future manufacture of orthodontic appliances.

1. Introduction

Several metallic materials are utilized in the daily practice of orthodontics. Stainless steel is present in wires, brackets and bands, and silver solder is usually the metal of choice to connect support wires in orthodontic appliances. Silver solder has been used extensively for this purpose due to some important advantages such as low cost and ease of use. Recently, work focusing on the biocompatibility of orthodontic materials has increased, and several studies have examined the release and cytotoxicity of orthodontic materials such as acrylic resins, composites and metals [1–8]. Biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy; it also refers to optimizing the clinically relevant performance of a given therapeutic intervention and generating the most appropriate beneficial cellular or tissue response in a given situation [8]. Corrosion is one of the major concerns relating to the issues surrounding the biocompatibility of metals [9,10]. Metallic ions may be released [2], leading to hypersensitivity and

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giving rise to allergic reactions, with local and systemic effects. Several auxiliary orthodontic appliances, such as lingual arches and maxillary expanders are made of stainless steel and contain silver soldered joints; these appliances may inhabit the oral cavity of a patient for months or even years. However, very few studies have investigated the safety and biocompatibility of the silver solder and the bands that are used in the preparation of orthodontic appliances [7,11–15]. Silver solder contains silver, copper and zinc as its major components [11]. These ions have a tendency to be released into the buccal cavity [16] and may give rise to cytotoxic events [3]. Although there have been a few investigations regarding the cytotoxic profile of silver solder [7,13–15], genotoxicity and DNA damage are still incipient themes in orthodontics. Comet and micronucleus assays have already been used to investigate the effects of stainless steel orthodontic appliances and archwires *in vitro* and *in vivo* [17–27]. However, to our knowledge, there has been no study concerning the evaluation of the potential genotoxic effects of stainless steel bands and bands with silver soldered joints. For this reason, the aims of this study were to evaluate the cytotoxic, cytostatic, genotoxic and oxidative DNA damage-inducing effects of stainless steel bands with or without silver soldered joints on HepG2 (human hepatocellular carcinoma) and HOK (human oral keratinocyte) cell lines and to quantify the ions released into the culture medium used to treat the cells.

2. Materials and methods

This study was approved by the ethics Committee of Pontifical Catholic University of Rio Grande do Sul (Porto Alegre, Brazil).

2.1. Evaluated materials

Stainless steel metallic orthodontic bands (universal bands for upper molars; Morelli, Sorocaba/SP, Brazil) were evaluated. According to the manufacturer's documentation, the bands are composed of 17–20% Cr, 8–10% Ni, and a maximum of 0.60% Mo and Fe. Two groups of bands were evaluated: silver soldered bands (SSB) and bands without any type of solder (non-soldered bands, NSB). The NSB group was composed of the bands evaluated as received from the manufacturer. For the silver solder group, a segment of stainless steel 1.0 mm wire (17–20% Cr, 8–10% Ni, and a maximum of 0.60% Mo and Fe) was soldered to the lingual side of each band using silver solder alloy (55–57% Ag, 21–23% Cu, 15–19% Zn and 4–6% Sn) and solder flux (Morelli, Sorocaba/SP, Brazil) heated by a butane micro-torch (GB 2001, Blazer, Farmingdale, NY, USA). The amount of solder alloy and flux and the polishing procedure were standardized because the soldering was executed by a single operator.

2.2. Chemicals

RPMI 1640 tissue-culture media, fetal bovine serum (FBS), trypsin-EDTA, L-glutamine and antibiotics were purchased from Gibco BRL (Grand Island, NY, USA). Oral Keratinocyte Medium (OKM), Oral Keratinocyte Growth Supplement (OKGS) and penicillin/streptomycin solution (P/S) were purchased from ScienCell Research Laboratories (CA, USA). 3-(4,5-Dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT), 5,5; hydrogen peroxide (H_2O_2), dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), cytochalasin B, Schiff's reagent and poly-L-lysine were purchased from Sigma (St. Louis, MO, USA). TrypLE Express, low-melting point agarose and agarose were obtained from Invitrogen (Carlsbad, CA, USA). Formamidopyrimidine-DNA glycosylase (FPG) and endonuclease III (EndoIII) were obtained from New England BioLabs (Beverly, MA, USA). Hydrochloric acid (HCl; 5 M) and DePex mounting medium were obtained from Merck (Darmstadt, Germany). All reagents were of analytical grade.

2.3. Preparation of metallic extracts

The experiments were preceded by the preparation of liquid extracts of each material to be investigated. For this, 12 bands of each group were first sterilized in an autoclave, as is usually done for clinical practice [28–30]. The bands were immersed in Falcon tubes containing 5 ml of RPMI 640 culture medium with 10% of serum for 24 h at 37 °C under agitation and were removed from the tube after this time. An aliquot of the culture medium was used to quantify the concentration of the ions eluted, and another aliquot was used to perform MTT and comet assays.

2.4. Assessment of ions eluted

To assess the concentrations of the ions eluted from the bands in RPMI 1640 media, atomic absorption spectrophotometry was employed. After the elution time (24 h), the bands were removed, and the media was analyzed for the presence of metallic eluates. Iron, nickel and chromium were quantified in both samples, while cadmium, copper, zinc and silver were quantified only in the SSB group. The culture medium alone was used as a blank. A flame atomic absorption spectrophotometer (SpectrAA 110, Varian, Palo Alto, CA, USA) was used to quantify copper, iron, silver and zinc. A graphite furnace atomic absorption spectrophotometer (ZEEnit 600, Analytik Jena, Jena, Thuringia, Germany) was used to quantify nickel, chromium and cadmium [9,11].

2.5. Cell culture

HepG2 is a human hepatocellular carcinoma cell line and was obtained from the ATCC (HB-8065). The choice of this cell line for this study was based on the fact that the HepG2 is a good experimental model system for the study of genotoxic agents, having a functionally active p53 protein, a competent DNA-repair system, active enzymes for phase-I and -II metabolism, and an active Nrf2 electrophile responsive system [31–33]. These properties tend to result in assays with a high predictivity for *in vivo* genotoxicity [34]. The cells were grown as monolayers under standard conditions in RPMI 1640 supplemented with 10% heat-inactivated FBS, 0.2 mg/ml L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. The cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere and were harvested by treatment with 0.15% trypsin-0.08% EDTA in PBS.

The Human Oral Keratinocyte (HOK) cell line was chosen to perform the CBMN-Cyt assay because this cell line provides a suitable model for cells of the buccal mucosa and has already been used to evaluate the effects of various metals [35]. The cell line was obtained from ScienCell Research Laboratories (Catalog number: 2610). This cell line was isolated from human oral mucosa and provides a good model to study basic keratinocyte biology as well as the processes of immortalization and malignant transformation. The cells were cultured in poly-L-lysine coated 25 cm^2 flasks, containing Oral Keratinocyte Medium, which is a complete medium for the optimal growth of normal human oral keratinocytes *in vitro* and is composed of basal medium plus a keratinocyte growth supplement and a penicillin/streptomycin solution. The cells were maintained in tissue culture flasks at 37 °C in a humidified 5%-CO_2 atmosphere and harvested by treatment with TrypLE Express.

2.6. MTT reduction assay

MTT reduction was performed as described by Denizot and Lang [36] for the HepG2 cell line. Two different treatment times were analyzed: 24 h and 3 h. Briefly, 1×10^4 cells per well were seeded in 96-well plates; after 24 h the cells were exposed for either 3 or 24 hrs to the NSB, SSB or negative control culture medium. The treatments were then washed out, and 150 μl of a 1 mg/ml MTT salt solution was added to each well. This assay is based on the ability of the mitochondrial enzyme succinate dehydrogenase to convert the yellow water-soluble tetrazolium salt (MTT) into formazan crystals in metabolically active cells [5]. After incubation for 3 h, the supernatant was removed, the obtained purple formazan product was re-suspended in 100 μL of Dimethyl Sulfoxide (DMSO), and the absorbance was read at 540 nm in a microplate reader (Enspire Multimode Plate Reader, Perkin Elmer, USA).

For the HOK cell line, 1×10^4 cells were seeded in 96-well plates coated with poly-L-lysine. After 24 h, the cells were exposed to the NSB, SSB or negative control culture medium for 24 h. After the treatment was washed out, fresh media with 10 μl of 5 mg/mL MTT salt solution was added to each well, and the plates were incubated for 4 h. Solubilizing solution (10% sodium dodecyl sulfate in 0.01 M HCl) was added to the plate and further incubated overnight at 37 °C in a humidified 5%-CO_2 atmosphere, and the absorbance was read at 570 nm with an ELISA microplate reader (SpectraMax 250, Molecular Devices, CA, USA).

2.7. Comet assay

2.7.1. Alkaline comet assay

The commonly used alkaline version of the comet assay detects DNA strand breaks and alkali labile lesions with high sensitivity [37]. The alkaline comet assay was performed as described by Singh et al. [38] with minor modifications [37,39]. Briefly 3×10^5 HepG2 cells were seeded in each well of 24 well-plates. After 24 h, the cells were exposed to the metallic eluates of NSB, SSB and negative control culture medium for 3 h or to a positive control (150 μM H_2O_2) for 2 h. After treatment, the cells were trypsinized and re-suspended in complete medium. Fifteen microliters of this cell suspension was mixed with 90 μl of 0.75% low-melting point agarose (LMP) and immediately spread onto a glass microscope slide pre-coated with a layer of 1.5% normal agarose. The LMP layer was allowed to set at 4 °C for 5 min, and the slides were incubated in ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH 10.0) at 4 °C for at least 1 h. This procedure removes the cell proteins and leaves the DNA as 'nucleoids'. Following lysis, the slides were placed on a horizontal electrophoresis unit and covered with fresh buffer (300 mM NaOH, 1 mM EDTA, pH 13.0) for 20 min at 4 °C to allow DNA unwinding and the expression of alkali labile sites. Electrophoresis was performed for 20 min at