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GRAZIELA HENRIQUES WESTPHALEN

CITOTOXICIDADE E GENOTOXICIDADE DE MATERIAIS ORTODÔNTICOS

Porto Alegre
2009

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Tese apresentada como parte dos requisitos obrigatórios para obtenção do grau de Doutor na área de Ortodontia e Ortopedia Facial pelo Programa de Pós-Graduação da Faculdade de Odontologia da Pontifícia Universidade Católica do Rio Grande do Sul.

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DEDICATÓRIA

A Deus, inteligência suprema, causa primária de todas as coisas.

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(Francisco Cândido Xavier - Chico Xavier)

RESUMO

A avaliação da biocompatibilidade dos materiais ortodônticos é de extrema importância, uma vez que elementos constituintes desses materiais estão relacionados a efeitos citotóxicos, genotóxicos e carcinogênicos Este estudo objetivou verificar a citotoxicidade e genotoxicidade de materiais ortodônticos. A citotoxicidade de materiais ortodônticos (bráquetes, fios, resinas compostas, soldas de prata e elásticos) foi testada por meio de duas exposições de sobrevivência ao *Saccharomyces cerevisiae (S. Cerevisiae)* de forma direta e indireta (produtos químicos liberados pelos materiais em saliva artificial) (Artigo 1). A avaliação da genotoxicidade foi realizada em grupos de pacientes, empregando-se os testes: Ensaio citoma bucal de micronúcleos para avaliação de aparelhos ortodônticos fixos e Hyrax (Artigo 2); Teste de micronúcleos para aparelhos expansores maxilares de Haas e Hyrax (Artigo 3). No artigo 1 foram realizadas comparações de ocorrência de sobrevivência de *S. Cerevisiae* em curvas semi-log entre os materiais ortodônticos e controle. Já nos artigos 2 e 3 foram empregados testes estatísticos não paramétricos diferentes ($\alpha \leq 0,05$) para comparações entre grupos de pacientes e controle. Foi observado que a solda de prata foi o único material que apresentou citotoxicidade. Todos os aparelhos testados não mostraram genotoxicidade nos grupos de pacientes estudados.

Palavras chaves: Materiais dentários, Ortodontia, Toxicidade, Gentoxicidade, Testes Mutagênicos, Teste de micronúcleos, *Saccharomyces cerevisiae,* Metais, Mucosa bucal, Célula basal.

ABSTRACT

Biocompatibility evaluation of orthodontic materials is very important, since some constituent elements of these materials are related to citotoxic, genotoxic and carcinogenic effects. This study aimed to assess citotoxicity and genotoxicity of orthodontic materials. Citotoxicity of orthodontic materials (brackets, wires, resin composites, solder silver and elastomers) was tested by two survival exposure to *Saccharomyces cerevisiae (S. Cerevisiae*), through direct and indirect (chemical products liberated by these materials in artificial saliva) forms (Article 1). Assessment of genotoxicity was carried out in patient groups, using the following tests: Cytome buccal micronucleus assay for evaluating orthodontic fixed appliances and Hyrax (Article 2), Micronucleus tests for Hyrax and Haas appliances (Article 3). For Article 1, occurrence of *S. Cerevisiae* survival was compared in a semi-log curve between orthodontic materials and control groups. For articles 2 and 3, non parametric test was used ($\alpha \leq 0.05\%$) for comparison among patients and control groups. Silver solder was the only material that showed citotoxicity. All appliances tested did not present genotoxicity in studied patients.

Key words: Dental Materials, Orthodontics, Toxicity, Genotoxicity, Mutagenic tests, micronucleus test, *Saccharomyces cerevisiae,* Metals, Buccal mucosa, Basal cell.

SUMÁRIO

1 INTRODUÇÃO

O aumento de pesquisas na área de biocompatibilidade dos materiais odontológicos é uma necessidade em função da capacidade dos mesmos provocarem alterações da atividade biológica nos tecidos envolvidos[1]. Esse fato está de acordo com uma tendência observada na ciência dos materiais dentários, de ser gradativamente substituída pela ciência dos biomateriais em Odontologia[2].

Determinados materiais odontológicos empregados na Ortodontia (ligas de aço inoxidáveis, solda de prata, resinas acrílicas) são constituídos de elementos químicos que estão relacionados a efeitos biológicos adversos no organismo humano[3-11].

Um dos aspectos da biocompatibilidade dos materiais odontológicos é a ação citotóxica dos mesmos. Muitos estudos[2,12-19] verificaram esse efeito, por meio de utilização de células de cultura humanas. Os resultados de tais estudos geraram conclusões diversas e conflitantes.

Uma metodologia que avalie com sucesso o potencial citotóxico se baseia no emprego de diversos organismos-modelo como *Saccharomyces cerevisiae (S. Cerevisiae)* que possibilita a realização de experimentos controlados e com um grande número amostral. No entanto, são escassas as pesquisas que utilizam esse método para avaliação dos efeitos citotóxicos relacionados aos materiais ortodônticos.

Outro aspecto, de fundamental importância na avaliação da biocompatibilidade de materiais ortodônticos, é o seu potencial genotóxico, uma vez que elementos metálicos como níquel, cromo, prata e cádmio apresentam resultados positivos relacionados à toxicidade genética[4,6,7,20-23]. Estudos *in vivo* mostraram que metais como o níquel e o cobalto, quando liberados dos aparelhos ortodônticos, podem causar quebras no DNA de células da mucosa bucal[3]. Apesar disso, ainda existem poucos estudos que abordem essa área de conhecimento.

A genotoxicidade pode ser avaliada por inúmeros experimentos, sendo abordada eficazmente pelos testes: teste de micronúcleos e ensaio citoma bucal de

micronúcleos. O teste de micronúcleos detecta danos genéticos tardios do DNA, por meio da verificação da freqüência de micronúcleos, que são estruturas extra-nucleares, compostas por cromossomos ou fragmentos dos mesmos que, durante a mitose, não foram incorporados ao núcleo principal[26,27]. O citoma bucal de micronúcleos possibilita uma avaliação mais abrangente, uma vez que além da verificação da freqüência de micronúcleos, avalia a presença de outros biomarcadores como: morte celular (cromatina condensada, cariorexe, cariólise e picnose), proliferação celular (núcleo duplo) e outros danos do DNA (nuclear *"BUDS"*) [28-30].

De acordo com o exposto, é de suma importância o conhecimento da capacidade que os materiais possuem de afetar o ambiente biológico, a fim de garantir um tratamento seguro e eficaz aos pacientes. Dessa forma, este estudo objetivou verificar a biocompatibilidade de alguns materiais ortodônticos, empregando teste de citotoxicidade *in vitro* utilizando *S. Cerevisiae,* como organismo modelo, bem como testes para avaliação da genotoxicidade como, ensaio citoma bucal de micronúcleos e teste de micronúcleos em um grupo de pacientes sob tratamento ortodôntico.

2 ARTIGO 1

Cytotoxicity of orthodontic materials assessed by survival tests in *Saccharomyces cerevisiae*

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Abstract

The aim of this study was to assess the cytotoxicity of orthodontic materials (brackets, wires, resin, elastomers and silver solder) using *Saccharomyces cerevisiae* as a model organism. The induction of cytotoxicity was assessed by two different tests using the wild-type *S. cerevisiae* strain FF18733: 1) direct exposure to orthodontic materials in YPD broth, and 2) exposure to artificial commercial saliva pre-treated with orthodontic materials. Only the silver solder was tested in mutant *S. cerevisiae* strains to investigate the origin of the observed cytotoxicity. Colony forming units per mL counts were carried out in all experiments and compared to control groups to detect significant survival differences. The results showed that only the silver solder induced significant cytotoxicity, which might have occurred via oxidative stress, although this mechanism is not completely understood.

Introduction

The biocompatibility of dental materials has been extensively studied [1, 2], since this property is essential to ensure the safe treatment of patients [3]. Some orthodontic materials, such as brackets, wires, solder and resins, have compounds known to have allergic, cytotoxic, mutagenic and/or carcinogenic potential [4-6]. These materials remain within the oral cavity for long periods and are subject to corrosion, which provokes the release of substances [7, 8] that might interact with patients' tissues [9].

The toxicity of some metals (iron, cooper, chromium, vanadium, cobalt, mercury, cadmium, nickel) might result from the generation of high levels of nitrogen and/or oxygen reactive species.[6]. Oxidative stress may cause oxidative damage in proteins, lipids and or/DNA and may trigger signalling cascades that stimulate cell growth [4].

Currently, many *in vitro* tests exist that can be used to assess the cytotoxicity of orthodontic materials, and various cell cultures are used that yield some similar and also some opposing findings [10-15]. Cytotoxicity induced by harmful agents can be assessed successfully by *in vitro* experiments using model microorganisms like the yeast *Saccharomyces cerevisiae* [16-18]. The use of this microorganism offers some advantages, since it is easy to cultivate and manipulate, and it provides quantitative and well-controlled experiments with large sample numbers and quick results. Moreover, as it is a eukaryotic microorganism, it is very similar to human and animal cells. However, few dental studies have used this microorganism for this purpose [19, 20].

The aim of this study was to evaluate the induction of cytotoxicity by orthodontic materials (brackets, wires, resin, elastomers and silver solder) using a wild-type *S. cerevisiae* strain as a model organism. Moreover, *S. cerevisiae* mutant strains were also used to investigate the origin of observed cytotoxicity induced by some of the orthodontic materials.

Materials and Methods

This cytotoxicity study was approved by the ethics Committee from Pontifícia Universidade Católica do Rio Grande do Sul, Brazil. The evaluation was performed using the orthodontic materials described in Table 1. These materials were all available for testing at the Clínica de Ortodontia, Pontifícia Universidade Católica do Rio Grande do Sul, Brazil.

S. cerevisiae strain, media and cultures

The *S. cerevisiae* strains used in this work and their respective genotypes are listed in Table 2. Included are the wild-type (WT) strain EG103 and its derivatives, which are single or double mutants in genes that encode the antioxidant defense enzymes Sod1, Sod2, and Cat1. Also included are the WT strain FF18733 and its derivatives, which are single, double or triple mutants in genes that encode the DNA base excision repair proteins Ogg1, Apn1, Apn2, Ntg1 and Ntg2.

The first experiments tested all the orthodontic materials but used only the WT strain FF18733. The other *S. cerevisiae* strains were only used to test the cytotoxicity of the solder fragments.

To cultivate all *S. cerevisiae* strains, YPD medium (1% yeast extract, 2% peptone, 2% glucose) was used, either in broth or solid (with agar at 2%) form. In all survival experiments, *S. cerevisiae* pre-cultures were prepared in 10 mL YPD broth and grown overnight to exponential phase (~10^7 cells/ mL) at 30°C.

Survival experiments for cytotoxicity analysis

The cytotoxicity analysis was performed via two types of survival experiments: 1- Direct exposure of *S. cerevisiae* cells to the orthodontic materials in YPD broth; 2 - Exposure to artificial commercial saliva (Salivan, Apsen Farmacêutica SA, Brazil) pre-treated with orthodontic materials.

For the direct exposure experiments, new inocula were made from the pre-cultures in 5 mL YPD, with each one containing pieces of different orthodontic materials, and a control without any material. These cultures were incubated at 30°C

to exponential phase (~10^{-7} cells/ mL). Aliquots from each culture were diluted in 0.9% sterile saline solution and 5-µL drops from each dilution (from 10^{-2} to 10^{-5}) were plated on YPD-agar and incubated at 30°C for two days for the emergence of small colonies, which allowed a qualitative approach. For quantitative analyses, 100 µL of the final dilutions were plated on YPD-agar (two plates for each dilution) for colony (CFU/mL) counting after two days at 30°C.

In saliva exposure experiments, the different orthodontic materials were immersed in 500 µL of artificial saliva for seven or twenty days. A total of 500 µL of the pre-inoculum was used for each treatment, which was centrifuged (2 min at 2000 g) and resuspended at 100% of saliva pre-exposed to the orthodontic materials. The cells were then treated for 60 minutes, diluted and plated in YPD-agar as described above, for both qualitative and quantitative analyses. A control with unexposed saliva was also done. At least three direct and three indirect experiments were performed with each type of orthodontic material.

Data analyses

The average Colony forming units per mL (CFU/mL) counts from three trials of each treatment were compared to their specific controls to verify the occurrence of significant survival differences in a semi-log curve (based on their standard deviation), which is an indication of cellular toxicity in *S. cerevisiae.*

Results

Direct exposure of *S. cerevisiae* strain FF18733 to orthodontic materials showed that the silver solder was the only material capable of inducing cytotoxicity, both qualitatively (Figure 1) and quantitatively (Figure 2). Silver solder promoted a significant reduction in CFU/ml values relative to the controls, and did so in a dose-dependent manner. Moreover, a complete inhibition of colony emergence was observed (Figure 3) in qualitative and quantitative tests with artificial saliva pre-exposed to three solder fragments (indirect), which confirms the high cytotoxic potential of the metals released from this orthodontic material. The other materials tested did not present any difference relative to the control groups.

The sensitivity to silver solder was next tested using *S. cerevisiae* strains deficient in genes that code for antioxidant enzymes and proteins that repair oxidative DNA damage; the results are summarized in Table 3. In tests of direct exposure to three, four and five solder fragments, no differences in terms of cell viability were observed for the mutant strains relative to the WT. In saliva exposure experiments, the EG133 strain, defective in superoxide dismutases 1 (CuZnSod, cytosolic) and 2 (MnSod, mitochondrial*)*, presented modest sensitivity (compared to WT strain EG103 in three experiments) to metals released by three, four or five solder fragments (Figure 4). The same occurred with strain BG1 (compared to WT strain FF18733 in three experiments), which is mutant for the AP-endonuclease 1 (Apn1) DNA repair protein. Nevertheless, the average values from three quantitative experiments with each mutant strain did not show significant differences compared to their respective wild-type strains (data not shown).

Discussion

In this study, several *S. cerevisiae* strains were used. First, one WT strain was used in an initial screen to assess the cytotoxic potential of all available orthodontic materials. Second, mutant strains deficient in DNA repair or antioxidant enzymes were used to investigate the basis of the cellular toxicity previously observed for the silver solder in the WT strain.

The results showed that brackets and wires (stainless steel alloys) did not induce any reduction in *S. cerevisiae* cell viability in either type of exposure. These findings are in line with several *in vitro* studies that evaluated the cytotoxicity of stainless steel orthodontic materials with different cell cultures [13-15]. However, some *in vivo* studies showed that orthodontic appliances composed of stainless steel alloys induced DNA damage in oral mucosa cells [21], and also increased the frequency of chromosomal breakage, as assessed via the micronucleus test [22]. This difference could be explained by the fact that *in vivo* experiments are difficult to control, since factors such as local irritants and adverse reactors might influence their results [23].

Moreover, an absence of cytotoxicity was also observed for the elastomers. Most studies have stated that these materials are mainly associated with allergic reactions [24, 25]. The same occurred for the light-cure resin, which corroborates the suggestion that this resin shows less cytotoxicity than auto-cure resin [26]. The data from most orthodontic materials that have been tested (brackets, wires, resin and elastomers) suggest that they bear considerable biocompatibility with *S. cerevisiae* cells and that they may be indicated for clinical use.

Significant cytotoxicity induced by silver solder was observed in this study, mainly in treatments with commercial saliva pre-exposed to solder fragments, with which a substantial decrease in *S. cerevisiae* cell viability was observed. Thus, this indicates that the artificial saliva promoted an intense metal release from the solder fragments during the seven- and twenty-day periods of exposure. In addition, this suggests that the use of silver solder may be injurious in the oral cavity environment.

The cytotoxic effects of silver solder have been clearly demonstrated by recent studies [11, 12, 27-30]. One aspect of cytotoxicity from silver solder can be attributed to its components (silver, copper, zinc, cadmium, nickel), which are known to have cytotoxic, genotoxic and carcinogenic effects [4-6]. Based on this information, several studies have presented laser solder as an alternative material with more biocompatibility for routine clinical use [27-29].

The experiments with the mutant strains suggested that oxidative stress may be involved in the cytotoxicity of silver solder. Two mutant strains defective in enzymes that defend cellular integrity during oxidative stress events (EG133 and BG1) showed differential sensitivity compared to WT strains when treated with saliva pre-exposed to three silver solder fragments. The EG133 strain is deficient in its two superoxide dismutases (CuZnSod and MnSod), which makes it extremely sensitive to the superoxide ions that are released at high levels during oxidative stress events [31]. The BG1 strain lacks the enzyme AP-endonuclease 1, which is involved in repairing oxidative damage in yeast nuclear and mitochondrial DNA. Thus, it is sensitive to environmental conditions that promote high levels of reactive oxygen species production [32]. These results agree with statements that recognize certain

metals, such as cadmium and cooper, as important promoters of oxidative stress [33, 34]. Since the mutant strains did not show any significant differences from the WT strains, further studies are necessary to better understand the basis of the cellular toxicity of silver solder.

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References

1. Wataha, J.C., *Principles of biocompatibility for dental practitioners.* J Prosthet Dent, 2001. **86**(2): p. 203-9.
2. St John, K.R., *Biocompatibility of dental materials.* Dent Clin North Am, 2007. **51**(3): p. 747-60, viii.
3. Montanaro, L., et al., *No genotoxicity of a new nickel-free stainless steel.* Int J Artif Organs, 2005. **28**(1): p. 58-65.
4. Beyersmann, D. and A. Hartwig, *Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms.* Arch Toxicol, 2008. **82**(8): p. 493-512.
5. Sethi, P.K. and D. Khandelwal, *Cadmium exposure: health hazards of silver cottage industry in developing countries.* J Med Toxicol, 2006. **2**(1): p. 14-5.
6. Valko, M., H. Morris, and M.T. Cronin, *Metals, toxicity and oxidative stress.* Curr Med Chem, 2005. **12**(10): p. 1161-208.
7. Amini, F., et al., *In vivo study of metal content of oral mucosa cells in patients with and without fixed orthodontic appliances.* Orthod Craniofac Res, 2008. **11**(1): p. 51-6.
8. Matos de Souza, R. and L. Macedo de Menezes, *Nickel, chromium and iron levels in the saliva of patients with simulated fixed orthodontic appliances.* Angle Orthod, 2008. **78**(2): p. 345-50.
9. Noort, R.V., *Introdução aos materiais dentários*. Vol. 2 edition. 2004, São Paulo: Artmed editora. 344.

10. Kao, C.T., et al., *The cytotoxicity of orthodontic metal bracket immersion media.* Eur J Orthod, 2007. **29**(2): p. 198-203.

11. Freitas, M.P., et al., *Cytotoxicity of silver solder employed in orthodontics.* Angle Orthod, 2009. **79**(5): p. 939-44.

12. Mockers, O., D. Deroze, and J. Camps, *Cytotoxicity of orthodontic bands, brackets and archwires in vitro.* Dent Mater, 2002. **18**(4): p. 311-7.

13. Assad, M., et al., *A new porous titanium-nickel alloy: Part 1. Cytotoxicity and genotoxicity evaluation.* Biomed Mater Eng, 2002. **12**(3): p. 225-37.

14. Assad, M., et al., *A new porous titanium-nickel alloy: part 2. Sensitization, irritation and acute systemic toxicity evaluation.* Biomed Mater Eng, 2002. **12**(4): p. 339-46.

15. Wever, D.J., et al., *Cytotoxic, allergic and genotoxic activity of a nickel-titanium alloy.* Biomaterials, 1997. **18**(16): p. 1115-20.

16. Perego, P. and S.B. Howell, *Molecular mechanisms controlling sensitivity to toxic metal ions in yeast.* Toxicol Appl Pharmacol, 1997. **147**(2): p. 312-8.

17. Avery, S.V., *Metal toxicity in yeasts and the role of oxidative stress.* Adv Appl Microbiol, 2001. **49**: p. 111-42.

18. De Freitas, J., et al., *Yeast, a model organism for iron and copper metabolism studies.* Biometals, 2003. **16**(1): p. 185-97.

19. Tanaka, H., et al., *Basic properties of an alginate impression material supplemented with chlorhexidine. I. Disinfectant effects on oral microbes.* J Nihon Univ Sch Dent, 1994. **36**(2): p. 135-8.

20. Williams, D.W., et al., *A novel technique for assessment of adherence of Candida albicans to solid surfaces.* J Clin Pathol, 1998. **51**(5): p. 390-1.

21. Faccioni, F., et al., *In vivo study on metal release from fixed orthodontic appliances and DNA damage in oral mucosa cells.* Am J Orthod Dentofacial Orthop, 2003. **124**(6): p. 687-93; discussion 693-4.

22. Westphalen, G.H., et al., *In vivo determination of genotoxicity induced by metals from orthodontic appliances using micronucleus and comet assays.* Genet Mol Res, 2008. **7**(4): p. 1259-66.

23. Brune, D., *Metal release from dental biomaterials.* Biomaterials, 1986. **7**(3): p. 163-75.

24. Levy, D.A., et al., *Allergy to latex.* Allergy, 1992. **47**(6): p. 579-87.

25. Conde-Salazar, L., et al., *Type IV allergy to rubber additives: a 10-year study of 686 cases.* J Am Acad Dermatol, 1993. **29**(2 Pt 1): p. 176-80.

26. Nocca, G., et al., *In vitro comparison of the cytotoxicity of two orthodontic composite resins.* Minerva Stomatol, 2006. **55**(5): p. 297-305.

27. Vande Vannet, B., J.L. Hanssens, and H. Wehrbein, *The use of three-dimensional oral mucosa cell cultures to assess the toxicity of soldered and welded wires.* Eur J Orthod, 2007. **29**(1): p. 60-6.

28. Sestini, S., et al., *In vitro toxicity evaluation of silver soldering, electrical resistance, and laser welding of orthodontic wires.* Eur J Orthod, 2006. **28**(6): p. 567-72.

29. Solmi, R., et al., *Interactions of fibroblasts with soldered and laser-welded joints.* Biomaterials, 2004. **25**(4): p. 735-40.

30. Grimsdottir, M.R., A. Hensten-Pettersen, and A. Kullmann, *Cytotoxic effect of orthodontic appliances.* Eur J Orthod, 1992. **14**(1): p. 47-53.

31. Gralla, E.B. and J.S. Valentine, *Null mutants of Saccharomyces cerevisiae Cu,Zn superoxide dismutase: characterization and spontaneous mutation rates.* J Bacteriol, 1991. **173**(18): p. 5918-20.

32. Ramotar, D., et al., *Cellular role of yeast Apn1 apurinic endonuclease/3'-diesterase: repair of oxidative and alkylation DNA damage and control of spontaneous mutation.* Mol Cell Biol, 1991. **11**(9): p. 4537-44.

33. Sinha, M., P. Manna, and P.C. Sil, *Induction of necrosis in cadmium-induced hepatic oxidative stress and its prevention by the prophylactic properties of taurine.* J Trace Elem Med Biol, 2009. **23**(4): p. 300-13.

34. Miller, C.D., et al., *Copper and cadmium: responses in Pseudomonas putida KT2440.* Lett Appl Microbiol, 2009.

Table 1: Description of orthodontic materials used.

Orthodontic material	**Composition**	**Fabricant**
Brackets and wires	Cobalt, chromium, nickel, trace amounts of molybdenum, beryllium, boron and carbon	American Orthodontics, Sheboygan, USA
Light-cure resin "Fill Magic Orthodontic"[1]	BisGMA, ester of methacrylic acid, glass of silicate fluoride	Vigodent, Rio de Janeiro, Brazil
Light-cure resin "Charisma"[1]	BisGMA, TeGMA, microglass (particle barium glass), silicon dioxide, light-cure initiators	Heraeus Kulzer, NY, USA
Elastomer	Natural latex – *Hevea brasiliensis, d*ietildtiocarbanato zinc, sulfur, zinc, silicon, cetostearyl alcohol, poly (vinyl-metril-eter)	Morelli, Sorocaba, Brazil
Silver Solder[2]	Silver, copper, cadmium, zinc and nickel	Morelli, Sorocaba, Brazil

[1] Fragments of resins were photoactived using the same light curing units (XL 3000, 3M Unitek – USA), following the recommendation of fabricants. All of them showed standard size and weight.

[2] Fragments of silver solder showed an average weight of 0.02 g each.

Table 2: Wild-type and mutant strains of *S. cerevisiae* used in survival experiments.

Wild-type and its derivative mutants in DNA repair	
Strain	**Genotype**
FF18733 (WT)	*mat a, ura3-52, his7-3, leu2-1, trp1-289, lys1-1*
BG1 (*apn1*)	like FF18733, *apn1::URA*
BG2 (*apn2*)	like FF18733, *apn2::kanMX*
BG3 (*apn1/apn2*)	like FF18733, *apn1::URA, apn2::kanMX*
CD138 (*ogg1*)	like FF18733, *ogg1::TRP1*
CD186 (*ogg1/ntg1/ntg2*)	like F18733, *ogg1::TRP1, ntg1::URA, ntg2::HIS*
Wild-type and its derivative mutants in antioxidant defense	
Strain	**Genotype**
EG103 (WT)	*mat a, leu2-3, 112 his3D1, trp1-289, ura3-52, GAL+*
EG118 (*sod1*)	like EG103, *sod1::URA3*
EG110 (*sod2*)	like EG103, *sod2:: TRP1*
EG133 *(sod1, sod2)*	like EG103, *sod1::URA3*, *sod2::URA3*
EG223 *(ctt1)*	like EG103, *ctt1:: TRP1*

Table 3: Summary of results from survival experiments with mutant *S. cerevisiae* strains exposed to silver solder fragments.

Experiment	Mutants in antioxidant enzymes	Mutants in DNA Repair enzymes
Direct Exposure	No differences compared to WT in any mutant strain	No differences compared to WT in any mutant strain
Exposure to Saliva	Some sensitivity observed for EG133 *(sod1, sod2)* strain	Some sensitivity observed for BG1 *(apn1)* strain

Figure 1: Qualitative analysis on YPD-agar from one direct exposure of (WT) *S. cerevisiae* strain FF18733 to various orthodontic materials. CN: control; SM= five solder fragments; CH: five "Charisma" resin fragments; FM: five "Fill Magic" resin fragments; EL: elastomer. Only the cells treated with SM fragments showed sensitivity compared to the control.

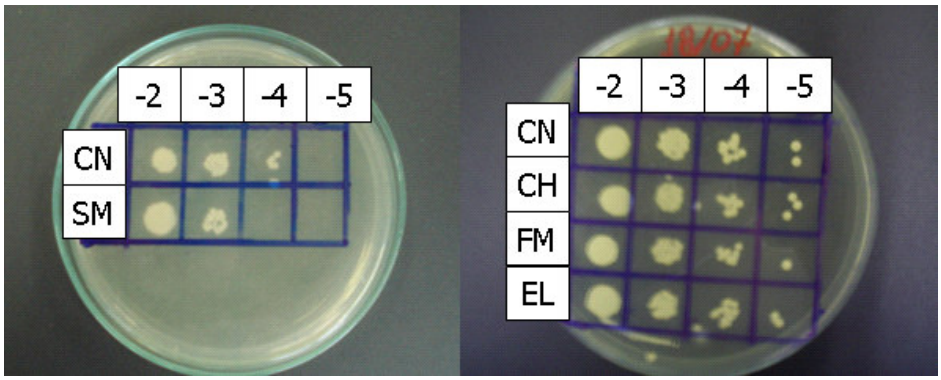


Figure 2: Average values (quantitative analysis) from three direct exposure experiments with *S. cerevisiae* strain FF18733 performed with three, four or five solder fragments in YPD-agar. Three SM= 0.075 g; four SM= 0.1 g; five SM= 0.125 g. The other orthodontic materials did not show any difference relative to the control groups (data not shown).

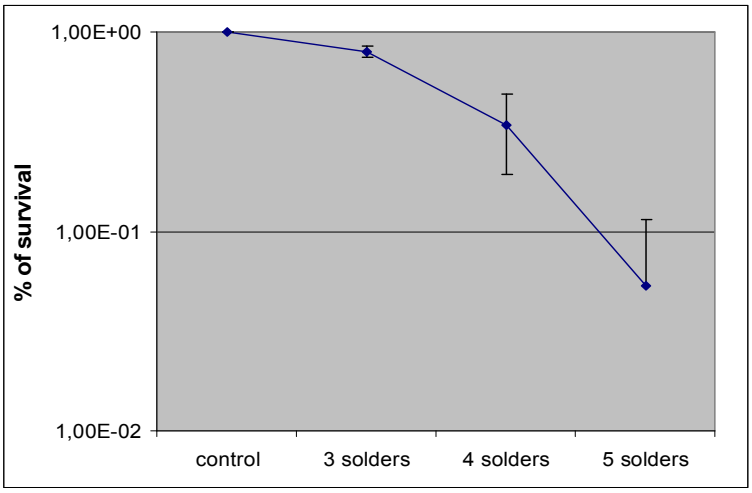


Figure 3: Qualitative analysis on YPD-agar from one exposure of (WT) *S. cerevisiae* strain FF18733 to saliva pre-treated with the different orthodontic materials. CN: control; SM= three solder fragments; CH: five “Charisma” resin fragments; FM: five resin “Fill Magic” fragments; EL: elastomers; BR: brackets. The SM fragments induced a complete inhibition of cell proliferation.

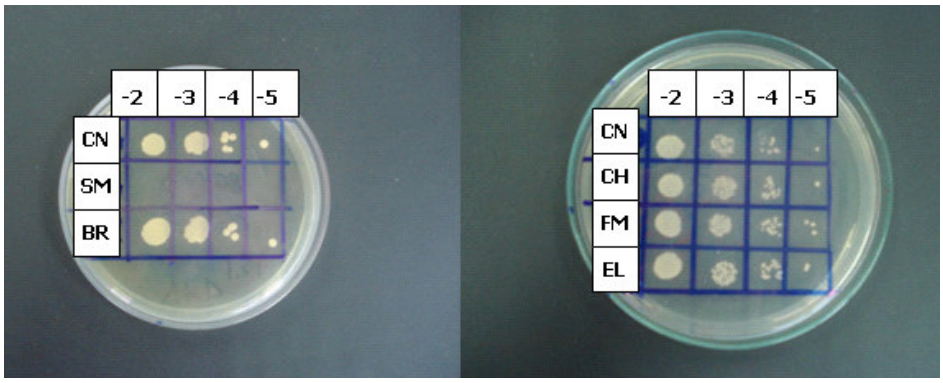
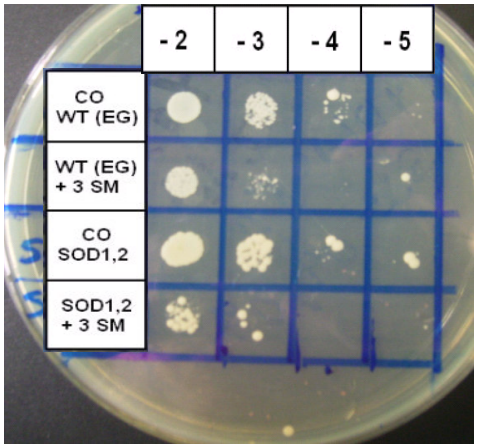


Figure 4: Qualitative analysis on YPD-agar from one exposure of *S. cerevisiae* strains EG103 (WT) and EG133 (*sod1/sod2)* to saliva pre-treated with three solder fragments (three SM), indicating a slight sensitivity of the mutant strain compared to the WT strain. A similar result was observed for BG1 (*apn1*) compared to the WT strain FF18733.



3 ARTIGO 2

Changes in Basal Cell Frequency in Orthodontic Patients

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Abstract

The Buccal mucosa of orthodontic patients is in long term contact with metals that might induce cytogenetic events. This study aimed to evaluate whether orthodontic patients have higher risk for abnormal cytogenetic events in buccal cells by investigating DNA damage, cell death and cell proliferation as determined by the buccal micronucleus cytome assay. The relationship and subsequent correlation between these various buccal cell types was also investigated. Buccal cell frequencies were compared among three randomized groups: Controls (n=31); patients wearing Hyrax appliance (n=30) and patients wearing fixed orthodontic appliances (n=32). No significant differences for cell death and DNA damage events were evident between control and study groups. Conversely, the frequency of basal cells was significantly reduced in groups fitted with both orthodontic appliances. Such differences might reflect alterations in cellular kinetics and the regenerative potential of buccal mucosa. Significant correlations are reflective of the inter-relationships of the various cell types.

Introduction

Orthodontic appliances are constantly subjected to corrosion processes (Goncalves *et al*., 2008), resulting in increased exposure of the buccal mucosa to the appliances' metal based components (Faccioni *et al*., 2003). These appliances consist of stainless steel alloys (35-65% cobalt; 20-30% chromium; 0- 30% nickel; trace amounts of molybdenum, silica, beryllium, boron and carbon) and welding (50% silver; 16% cadmium; 16% copper; 15% zinc; 3% nickel) and are usually worn for an average mean time of 2 years. It is known that some of these metals such as: nickel, chromium and cadmium exhibit both carcinogenic and mutagenic potential (Beyersmann and Hartwig, 2008; Valko *et al*., 2005). Therefore, it is important to investigate the potential cytotoxicity effects of these appliances on cells of the buccal mucosa.

The buccal micronucleus cytome (*BMCyt*) assay is a minimally invasive and established method for monitoring cytogenetic damage in human epithelial cells (Fenech *et al*., 2007; Holland *et al*., 2008; Thomas *et al*., 2009). Its application involves the assessment of biomarkers for DNA damage (micronuclei *(MNi*) and nuclear bud (*NBUD*) frequency), Cell proliferation rates (basal and binucleated cells) and Cell Death parameters (karyorrhexis, condensed chromatin, karyolitic and pyknotic cells). Potential abnormal cytogenetic effects from orthodontic appliances have not been previously assessed using the *BMCyt* assay. Previous studies investigating these effects include: evaluation of *MNi* frequency (Westphalen *et al*., 2008), comet assay sensitivity (Faccioni *et al*., 2003) and several *in vitro* cell culture studies (Assad *et al*., 2002a; b; Montanaro *et al*., 2005; Wever *et al*., 1997). The aims of this study was to investigate whether orthodontic patients have a higher risk for abnormal cytogenetic effects in buccal cells by investigating cell proliferation rates, DNA damage and cell death parameters as determined by the *BMCyt* assay and also to evaluate the correlation and subsequent inter-relationships between these DNA biomarkers and cells parameters.

Materials & methods

Approval for this study was obtained from the Pontifícia Universidade Católica do Rio Grande do Sul, Brazil review board. The participant's rights have been explained and informed consent obtained. Frequency of *BMCyt* assay parameters was measured on buccal cells of individuals, at University cited, from three groups (randomized), by one operator: 1) Control (n=32, age: 9.03± 1.04 years, gender: 18 males/14 females) consisting of individuals without metal dental fillings; 2) Hyrax appliance (n=31, age: 11.3± 1.93 years, gender: 17 males/14 females) with patients wearing Hyrax appliance (Composition: stainless steel alloy and welding) for six months and 3) Fixed orthodontic appliance (n=30, age: 22.0± 9.08 years, gender: 13 males/17 females) composed of patients with fixed orthodontic appliance (Composition: stainless steel alloy) for 24 months. Individuals with certain lifestyle factors (*i.e.* smoking or excessive alcohol intake), or occupational profiles (*i.e.* exposure to metal ions or metal dental fillings), which has been shown to influence genomic stability events were excluded.

Cell sampling and preparation of slides for microscopic analysis followed the protocol: "Buccal micronucleus cytome assay" described by Thomas, Holland et al. 2009. Slides were de-identified and scored using a microscope equipped with a triple-band filter (Nikon E600, Tokyo, Japan) at x1000 magnification (Dapi, FITC and Rhodamine) in blind manner. Cells containing *MNi* on bright field were confirmed as being positive by examining the cells under fluorescence (Figure 1). First, frequency of all the various cell types was determined in 1000 cells and afterwards, frequency of DNA damage biomarkers (*MNi* and *NBUD*) was determined in 2000 differentiated cells and 200 basal cells. Figure 1 illustrates the different cells scored in the *BMCyt* assay whilst more detailed descriptions are outlined below.

Normal basal cells (Figure 1a)

These cells are derived from the basal layer. The nuclear to cytoplasmic ratio is larger than that in differentiated buccal cells. When compared to differentiated cells, basal cells are smaller with a uniformly stained nucleus with more intense green cytoplasmic stain.

Normal differentiated cells (Figure 1b)

These cells are derived from basal cells and have smaller nuclear to cytoplasmic ratio relative to basal cells. The nucleus is oval or round, uniformly stained and generally has lighter cytoplasmic green stain compared to basal cells.

Cells with micronuclei (Figure 1c and d)

These cells are characterized by the presence of one or two smaller *MNi*, which are biomarkers of whole chromosome loss or breakage because they originate from whole chromosomes or fragments that lag behind at anaphase (Fenech *et al*., 1999). They range in size from 1/3 to 1/16 of the diameter of main nucleus, with a similar staining intensity and texture to main nucleus.

Cells with Nuclear Bud (Figure 1e)

Cells containing a nuclear bud have a main nucleus with an apparent sharp constriction indicative of a budding process. The *NBUD* is similar to the main nucleus in terms of morphology and staining intensity. However, buds are smaller and range in diameter size from half to quarter of that of the main nucleus.

Binucleated cells (Figure 1f)

These cells contain two distinct nuclei that are uniformly stained and sometimes appear to be touching. These cells may be indicative of nuclear division rate and/or failed cytokinesis following the last nuclear division.

Condensed chromatin cells (Figure 1g)

This cell population possesses nuclei with distinctive regions of condensed or aggregated chromatin, exhibiting a speckled or striated pattern. Some areas of the chromatin are darkly stained whereas others appear lighter due to loss of nuclear material.

Karyorrhectic cells (Figure 1h)

Karyorrhectic cells exhibit more extensive nuclear chromatin aggregation relative to condensed chromatin cells, leading to nuclear fragmentation and eventual disintegration.

Pyknotic cells (Figure 1i)

These cells are characterised by a small shrunken nucleus with a diameter usually one to two thirds that of a normal nucleus. The nuclear material contained within pyknotic cells is uniformly and intensely stained.

Karyolytic cells (Figure 1j)

These cells when stained with Schiffs and Light Green appear negative for nuclear material when viewed under both light and fluorescence microscopy. These cells appear to have no nucleus although the faint outline of a membrane can sometimes be determined.

Statistical analysis

One-way Analysis of Variance (Kruskal-Wallis test) followed by Dunn's Multiple Comparison test was applied to assess the significance of the cellular and nuclear parameters measured between cohorts and Mann-Whitney test was applied to evaluate gender and age differences. Correlations between biomarkers were tested using Spearmann test ($P \leq 0.05$). These analysis values were performed using SPSS version 10.0 (SPSS Inc, Chicago, IL) and Prism 5.0 (GraphPad Software Inc, San Diego, CA).

Results

There were no significant differences in the frequency of DNA damage rates, cell death and proliferation parameters among the three cohorts (Table 1).Only basal cell frequency was significantly reduced in hyrax (19%) and fixed orthodontic (25%) appliances relative to the control (p=0.022; Table 1; Figure 2). No age or gender

differences were observed for any parameter evaluated. Correlation analysis between DNA biomarkers and cell parameters are described in Table 2.

Discussion

Previous studies have shown that the amount of metal ions liberated from orthodontic appliances into the oral cavity due to corrosion processes was significantly below the average dietary intake and did not reach toxic concentrations (Agaoglu et al., 2001; Kocadereli et al., 2000). However, it can not be excluded that even presumed non-toxic concentrations of these metals may be sufficient to induce biological effects *in vivo* within oral mucosa cells over the long term chronic exposure period (Faccioni et al., 2003). The aim of the current study was to assess the potential cytotoxic effects which may result from orthodontic appliances using the *BMCyt* assay. This is the first time that this assay has been used to investigate the potential cytotoxic effects from orthodontic appliances. This minimally invasive assay allows the assessment of biomarkers that are reflective of DNA damage events, cell proliferation and cell death parameters (Fenech et al., 2007; Holland et al., 2008; Thomas et al., 2009). Buccal cells can be easily accessed and reflect target sites that have been shown to be sensitive biomarkers to the insults imposed by genotoxic events, environmental and occupational exposures (Burgaz et al., 1999; Sarto et al., 1990; Stich et al., 1984)

The results of this research showed that the use of appliances (Hyrax and fixed orthodontic) were not related to any increase in cytotoxic events (Table 1), which are in line with findings from several *in vitro* trials (Assad et al., 2002a; b; Montanaro et al., 2005; Wever et al., 1997). In the same way, the occurrence of these effects could not be confirmed through a trial that used both the *MNi* frequency and comet assay in a group of fixed orthodontic patients, because both assays showed opposing results (Westphalen et al., 2008). However, other studies have shown that nickel and cobalt released from fixed orthodontic appliances in groups of patients could induce DNA damage in oral mucosa cells (Faccioni et al., 2003). Laboratory technicians exposed to metals (chromium, cobalt and nickel) resulting from being in contact with dental materials showed an increase in the Micronucleus

(*MN*) frequency in peripheral lymphocytes and exfoliated nasal cells (Burgaz et al., 2002). However, our results do not show a marked difference between the study groups and controls for *MN* frequency or nuclear buds. As expression of *MNi* require nuclear division, *MN* frequency in buccal mucosa is also dependent on the proportion of once divided cells. It is possible that the reduced number of basal cells and perhaps a reduced proportion of actively dividing basal cells in the appliance groups may inhibit *MNi* and *NBUD* expression (the latter being S-phase dependent) (Shimizu et al., 1998). The differences in these studies are probably attributable to the differing kinetics of the basal and differentiated cells.The only parameter that was significantly different between the control and study groups was the frequency of basal cells, ($p=0.022$). This may be reflective of a reduced regenerative potential resulting from metal alloy exposure which could lead to altered cell kinetics and changes within the structural profile of the buccal mucosa as well as *MNi* and *NBUD* expression. The changes in basal cell frequency between the three cohorts are also thought not to reflect any age related changes, given the fact age was not associated to any evaluated parameter, possibly because the age similarity between subjects. It has been shown previously that the basal cell frequency tends to increase with normal ageing but has been found to be significantly reduced in examples of premature ageing syndromes such as Alzheimer's and Down's syndrome (Thomas and Fenech, 2007; Thomas et al., 2008).

Cross correlation analyses of biomarkers from this study (Table 2) show that they are in agreement with the data of Thomas et al (2008) regarding the positive association between condensed chromatin and karyorrhexis, pyknosis or basal cells. Conversely to the study of Thomas et al (2008) with Down syndrome subjects and young or old controls, we observed a negative correlation between condensed chromatin and karyolysis and did not observe correlations between basal and karyorrhexis nor between pyknosis and karyorrhexis. Based in the assumption that the correlations are indicative of the biological association between the cell types and their likely sequential development (Thomas et al, 2008) and that the present study is one of the first to evaluate buccal cell dynamics, we can provide some early input about cell dynamics in the oral mucosa. For instance, the positive correlation

between basal and condensed chromatin indicate the later cells might derive directly from basal cells. The negative correlation between karyolysis and condensed chromatin or basal cells indicate karyolitic cells are not likely to derive from basal or condensed chromatin cells in healthy subjects. The high correlation between karyolysis and differentiated cells indicate the former cells originate from the latter. Further studies are needed to better understand buccal cell dynamics both in healthy and/or pathological situations.

One of the limitations of this study was the difference in age amongst the three groups. This occurred because each group requested specific characteristics e.g.: for the control it was necessary to have individuals with no metal dental fillings within the oral cavity.; Hyrax appliance is mainly used in children and young adults, whereas the fixed orthodontic appliance is usually worn in older individuals. The mean time of use for both appliances could not be accurately matched as each appliance presents determined time of use. Control group also presented high frequency of *NBUD*'s per 2000 differentiated cells. Although it is tentative to explain differences in DNA damage background due to socioeconomic and lifestyles characteristics, however both controls and individuals fitted with appliances showed similarities, since both lived in low income areas. Another aspect of this study was that it evaluated only the target cells, it is encouraged that surrogate tissue and systemic metal levels are assessed (Burgaz *et al*., 2002), in order to provide an overall biological effect of appliance in the individuals.

In conclusion, this study suggested a reduction in the regenerative potential of this tissue possibly reflecting changes in cellular kinetics and the structural profile of the buccal mucosa.

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Brazil, especially Fabiane Azeredo for helping to recruit the individuals and to those individuals who participated voluntarily in this study.

References

Agaoglu G, Arun T, Izgi B, Yarat A (2001). Nickel and chromium levels in the saliva and serum of patients with fixed orthodontic appliances. *Angle Orthod* 71:375-9.

Assad M, Chernyshov A, Leroux MA, Rivard CH (2002a). A new porous titanium-nickel alloy: part 2. Sensitization, irritation and acute systemic toxicity evaluation. *Biomed Mater Eng* 12:339-46.

Assad M, Chernyshov A, Leroux MA, Rivard CH (2002b). A new porous titanium-nickel alloy: Part 1. Cytotoxicity and genotoxicity evaluation. *Biomed Mater Eng* 12:225-37.

Beyersmann D, Hartwig A (2008). Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. *Arch Toxicol* 82:493-512.

Burgaz S, Karahalil B, Bayrak P, Taskin L, Yavuzaslan F, Bokesoy I, *et al.* (1999). Urinary cyclophosphamide excretion and micronuclei frequencies in peripheral lymphocytes and in exfoliated buccal epithelial cells of nurses handling antineoplastics. *Mutat Res* 439:97-104.

Burgaz S, Demircigil GC, Yilmazer M, Ertas N, Kemaloglu Y, Burgaz Y (2002). Assessment of cytogenetic damage in lymphocytes and in exfoliated nasal cells of dental laboratory technicians exposed to chromium, cobalt, and nickel. *Mutat Res* 521:47-56.

Faccioni F, Franceschetti P, Cerpelloni M, Fracasso ME (2003). In vivo study on metal release from fixed orthodontic appliances and DNA damage in oral mucosa cells. *Am J Orthod Dentofacial Orthop* 124:687-93.

Fenech M, Holland N, Chang WP, Zeiger E, Bonassi S (1999). The HUman MicroNucleus Project--An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. *Mutat Res* 428:271-83.

Fenech M, Crott JW (2002). Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes-evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay. *Mutat Res* 504:131-6.

Fenech M, Bolognesi C, Kirsch-Volders M, Bonassi S, Zeiger E, Knasmuller S, et al. (2007). Harmonisation of the micronucleus assay in human buccal cells--a Human Micronucleus (HUMN) project (www.humn.org) initiative commencing in 2007. *Mutagenesis* 22:3-4.

Gonçalves TS, de Menezes LM, Silva LE (2008). Residual monomer of autopolymerized acrylic resin according to different manipulation and polishing methods. An in situ evaluation. *Angle Orthod* 78*:*722-7.

Holland N, Bolognesi C, Kirsch-Volders M, Bonassi S, Zeiger E, Knasmuller S, *et al*. (2008). The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps. *Mutat Res* 659*:*93-108.

Kocadereli L, Atac PA, Kale PS, Ozer D (2000). Salivary nickel and chromium in patients with fixed orthodontic appliances. *Angle Orthod* 70*:*431-4.

Montanaro L, Cervellati M, Campoccia D, Prati C, Breschi L, Arciola CR (2005). No genotoxicity of a new nickel-free stainless steel. *Int J Artif Organs* 28*:*58-65.

Sarto F, Tomanin R, Giacomelli L, Iannini G, Cupiraggi AR (1990). The micronucleus assay in human exfoliated cells of the nose and mouth: application to occupational exposures to chromic acid and ethylene oxide. *Mutat Res* 244*:*345-51.

Shi Q, King RW (2005). Chromosome nondisjunction yields tetraploid rather than aneuploid cells in human cell lines. *Nature* 437*:*1038-42.

Shimizu N, Itoh N, Utiyama H, Wahl GM (1998). Selective entrapment of extrachromosomally amplified DNA by nuclear budding and micronucleation during S phase. *J Cell Biol* 140*:*1307-20.

Stich HF, Rosin MP, Vallejera MO (1984). Reduction with vitamin A and beta-carotene administration of proportion of micronucleated buccal mucosal cells in Asian betal nut and tobacco chewers. *Lancet* 1*:*1204-6.

Thomas P, Fenech M (2007). A review of genome mutation and Alzheimer's disease. *Mutagenesis* 22*:*15-33.

Thomas P, O'Callaghan N, Fenech M (2008). Telomere length in white blood cells, buccal cells and brain tissue and its variation with ageing and Alzheimer's disease. *Mech Ageing Dev* 129*:*183-90.

Thomas P, Holland N, Bolognesi C, Kirsch-Volders M, Bonassi S, Zeiger E, *et al*. (2009). Buccal micronucleus cytome assay. *Nat Protoc* 4*:*825-37.

Valko M, Morris H, Cronin MT (2005). Metals, toxicity and oxidative stress. *Curr Med Chem* 12*:*1161-208.

Westphalen GH, Menezes LM, Pra D, Garcia GG, Schmitt VM, Henriques JA, *et al*. (2008). In vivo determination of genotoxicity induced by metals from orthodontic appliances using micronucleus and comet assays. *Genet Mol Res* 7*:*1259-66.

Wever DJ, Veldhuizen AG, Sanders MM, Schakenraad JM, van Horn JR (1997). Cytotoxic, allergic and genotoxic activity of a nickel-titanium alloy. *Biomaterials* 18*:*1115-20.

Table 1. Frequency of DNA damage, cell death and cell proliferation parameters among three cohorts.

Parameter	**Group**			*p*
	Control (n=31)	**Hyrax appliance (n=30)**	**Orthodontic fixed appliance (n=32)**	
DNA damage				
DNA damage MNi / 2000 diff cells	0.00 ± 0.00	0.10 ± 0.40	0.13 ± 0.35	0.110
DNA damage NBUD / 2000 diff cells	2.41 ± 2.01	2.68 ± 2.37	2.50 ± 2.71	0.836
DNA damage MNi / 200 basal cells	0.00 ± 0.00	0.03 ± 0.18	0.00 ± 0.00	0.368
DNA damage NBUD / 200 basal cells	0.09 ± 0.30	0.29 ± 1.44	0.03 ± 0.18	0.635
Cell death				
Karyorrhexis /1000 cells	0.19 ± 0.59	0.19 ± 0.48	1.87 ± 7.35	0.437
Pyknotic /1000 cells	21.13 ± 11.55	21.32 ± 14.04	25.40 ± 16.88	0.615
Karyolysis /1000 cells	57.13 ± 48.79	87.35 ± 63. 67	73. 50 ± 65.62	0.129
Condensed Chromatin /1000 cells	4.63 ± 8.96	2.61 ± 3.70	3.63 ± 4.75	0.596
Cell proliferation				
Binucleates /1000 cells	0.97 ± 1.53	0.58 ± 0.99	0.93 ± 1.28	0.380
Basal cells /1000 cells	72.97 ± 35.14	59.03 ± 48.18	51.80 ± 25.70	0.022*
Differentiated cells /1000 cells	841.91 ± 61.51	831.45 ± 64.46	842.40 ± 64.91	0.801

p: level of significance according to Kruskal-Wallis test. * Significant difference.

Table 2. Cross-correlation results between biomarkers of the buccal cytome assay for combined cohorts (*N*=93). Negative correlation are represent by negative symbol, whereas positive correlation has no symbol

		MN in diffentiated cells	NBud in diffentiated cells	MN in basal cells	NBud in basal cells	Karyorrhexis	Pyknosis	Binucleates	Karyolysis	Condensed Chromatin	Basal cells
NBud in diffentiated cells	Spearmann Correlation Sig. (2-tailed)	0.314 0.004									
MN in basal cells	Spearmann Correlation Sig. (2-tailed)	0.421 <0.001	NS								
NBud in basal cells	Spearmann Correlation Sig. (2-tailed)	NS	NS	NS							
Karyorrhexis	Spearmann Correlation Sig. (2-tailed)	NS	NS	NS	NS						
Pyknosis	Spearmann Correlation Sig. (2-tailed)	NS	NS	NS	NS	NS					
Binucleates	Spearmann Correlation Sig. (2-tailed)	NS	NS	NS	NS	NS	0.223 0.043				
Karyolysis	Spearmann Correlation Sig. (2-tailed)	NS	-0.374 <0.001	NS	NS	NS	NS	-0.273 0.012			
Condensed Chromatin	Spearmann Correlation Sig. (2-tailed)	NS	NS	NS	NS	0.350 0.001	0.261 0.017	0.271 0.013	-0.221 0.013		
Basal cells	Spearmann Correlation Sig. (2-tailed)	NS	NS	NS	NS	NS	NS	NS	-0.356 <0.001	0.254 0.021	
Differentiated cells	Spearmann Correlation Sig. (2-tailed)	NS	NS	NS	-0.262 0.016	NS	NS	NS	0.680 <0.001	NS	-0.267 0.015

Figure 1. Different cell types scored in buccal cytome assay in both bright and fluorescence field. a) Normal basal cells; b) Normal differentiated cells; c) and d) Cells with micronuclei, arrows show these structures; e) Cell with nuclear bud; f) Binucleated cells; g) Condensed chromatin cells; h) Karyorrhectic cells; i) Pyknotic cells; j) Karyolytic cells.

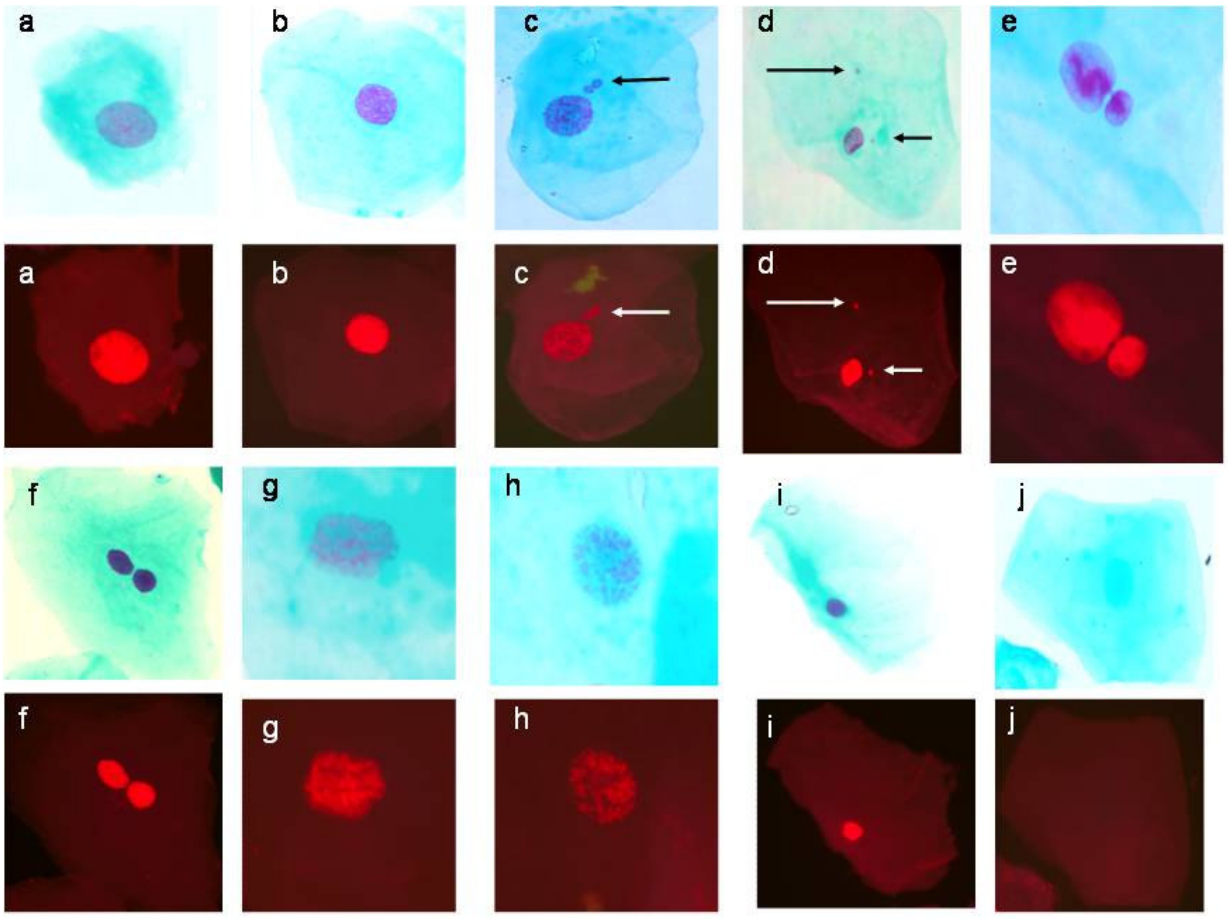
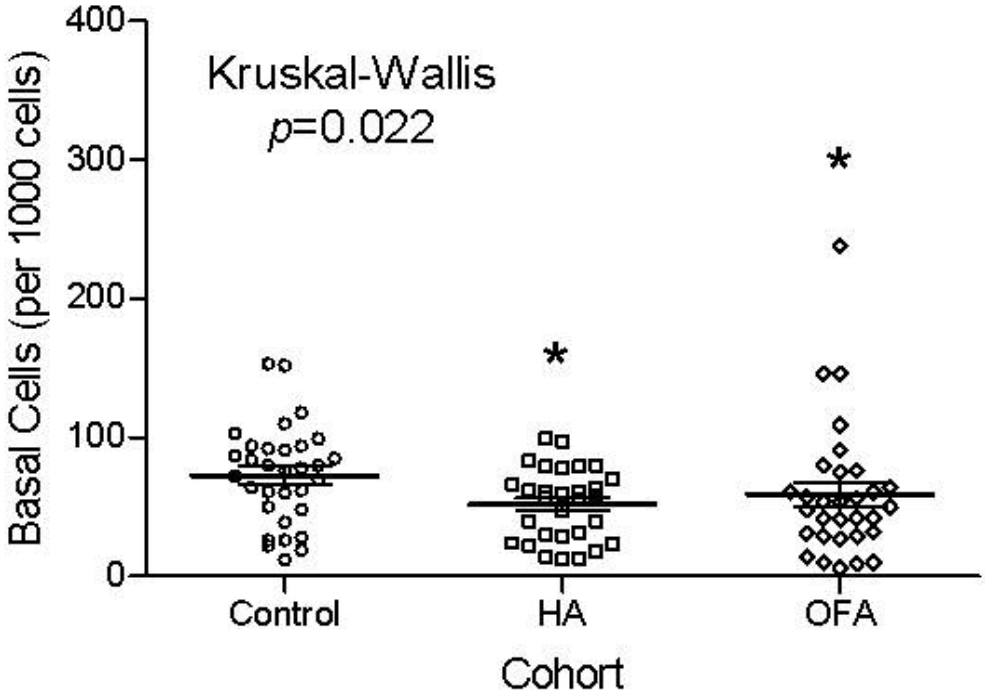


Figure 2. Reduction of basal cell frequency in individuals wearing appliances. *statistical difference in relation to control according to Dunn's Multiple Comparison test. HA: Hyrax appliance; OFA: Orthodontic fixed appliance.



4 ARTIGO 3

In vivo genotoxicity assessment of maxillary expander appliances

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Abstract

Some components of maxillary expansor appliances (Haas and Hyrax) might induce genotoxic effects. The aim of this study was to assess the genotoxicity related to wearing maxillary expansor appliances (Haas and Hyrax) in group of patients. Micronucleus assays were carried out in buccal cells, which were sampled before (T1), 10 (T2) and 30 (T3) days after the placement of maxillary expansor appliances. The frequency of micronuclei was compared among three periods, using the Wilcoxon test ($p<0.05$). Results showed that compared with T1, the micronucleus frequency decreased significantly at T3. Therefore, the appliances did not show any genotoxic effects in these patients. However, further studies in long-term models using different methodologies are necessary to better understanding the biological effects.

Introduction

Negligence of biocompatible dental materials can provoke health risks to patients, dental staff members and practitioners themselves [1]. Maxillary expander appliances might be constituted by stainless steel alloys (35-65% cobalt; 20-30% chromium; 0- 30% nickel; small amount of molybdenum, silicon, beryllium, boron and carbon), welding (50% silver; 16% cadmium; 16% copper; 15% zinc; 3% nickel) and acrylic resins (methacrylates), which contain elements with known genotoxic potential [2-4]. When these appliances are present in the oral cavity, their constituents elements can be released by corrosion [5]. Therefore, it is important to study the biological effects of these appliances.

The study of genotoxicity is a field of genetics that studies the processes that alter heredity (mutagenesis) or genetic determinism (carcinogenesis and teratogenesis) [6]. There is a wide range of assays to verify genotoxicity; one of them is the micronucleus (MN) assay, which has been successfully used to monitor human populations exposed to mutagenic and carcinogenic agents [7]. This assay evaluates the frequency of MN, which are extracellular structures composed of chromosomes or chromosome fragments that are not incorporated into the main nucleus during mitoses [8].

Concerning the importance of biocompatibility, this study used the MN assay to assess the genotoxicity related to wearing of maxillary expansor appliances (Haas and Hyrax) in a group of patients.

Materials and methods

This study was approved by the Pontifical Catholic University of Rio Grande do Sul Ethics Committee. Ten patients (mean age:12.2 ± 6.9 years; 6 males and 4 females) undergoing maxillary expansion with two appliances, Haas (stainless steel alloy and acrylic resin) (n=7) and Hyrax (stainless steel alloy and welding) (n= 3), participated in this study. Individuals with certain lifestyle factors (*i.e.,* smoking or excessive alcohol intake), or certain occupational profiles (*i.e.,* exposure to metal

ions or metal dental fillings) that have been shown to influence genomic stability were excluded.

Buccal cell samples were collected by one operator from patients at three periods: before (T1), 10 days (T2) and 30 (T3) days after placement of appliances. The patients rinsed with distilled water for 2 minutes to remove exfoliated buccal cells [9]; cells were collected using swabs and transferred to polyethylene tubes (50 ml) containing phosphate-buffered saline (20 ml) and slides were prepared according to the protocol established by Titenko-Holland, Moore and Smith (1994) [10].

Slides were analyzed at 1000X magnification in oil immersion with light microscope (Axiolab, Zeiss) to determine the frequency of MN; 1000 cells were evaluated for each patient. Only cells that were not smeared, clumped or overlapping and that contained intact nuclei were included in the analysis. MN were identified if they had the following characteristics: less than 1/3 diameter of the main nucleus; the same plane of focus; the same color, texture and refraction as the main nucleus; smooth oval or round shape, and clearly separated from the main nucleus [8].

MN frequency was compared among the three periods, using Wilcoxon test ($p<0.05$ was considered statistically significant) with SPSS version 10.0 (SPSS Inc, Chicago, USA). All values are expressed as mean ± standard deviation.

Results

There was a significant difference in MN frequency only for the comparison between T1 and T3 (Table I), where a significant decrease of MN frequency was observed at T3 (Figures 1 and 2).

Discussion

The scope of this study was to verify the genotoxicity of the maxillary expander appliances, since they have constituents with known genotoxic effects and might stay in oral cavity for a median period of 6 months, often in young patients whose organs are developing.

The MN assay is an *in vivo* assay often used to detect whether agents have caused chromosomal damage [7]. MN formation is an effective biomarker of

illness and the process associated with induction of DNA damage [7, 8, 11, 12]. MN occurrence is detected after cellular division and after some time has passed from exposure to a harmful agent [13]. Thus, in this study, buccal cells were sampled at 10 (T2) and 30 (T3) days after placement of appliances. The type of cells used (buccal) was successful for using the MN assay, as it requires minimally invasive procedures for sampling [7, 12, 14], and it also constitutes the target cells, which are in direct contact with the appliances.

This study showed that the maxillary expander appliance did not provoke genotoxic effects in the sample of patients. These findings are in line with several *in vitro* cell culture studies [15-18]. Genotoxicity effects from fixed orthodontic appliances were not confirmed by Westphalen et al. [19], however, an increase of MN was observed after 30 days of placement of the appliance evaluated. In contrast, Faccioni et al. [20] found that metals (nickel and cobalt) released from fixed orthodontic appliances induced DNA damage to the buccal cells of orthodontic patients. Burgaz et al. [21] observed that metals ions released by stainless steel alloys (chromium, cobalt and nickel) were associated with increases in MN frequency in both lymphocytes and nasal cells of dental technician staff.

One of the limitations of this study was the small sample size, due to patient´s desistence of study´s step (sampling). In addition, there was evaluation of only buccal cells. Burgaz et al. [21] suggested that beyond the target cells, other cells (e.g., lymphocytes) should also be analyzed and systemic measures should be carried out to assess genotoxicity from dental materials.

Conclusion

The maxillary expander appliances (Haas and Hyrax) did not induce genotoxic damage in the population evaluated. However, it is necessary that long-term studies be conducted, using a wide-range of tests and systemic measures, to better assess the full effect.

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References

1. Wataha JC. Principles of biocompatibility for dental practitioners. J Prosthet Dent 2001;86:203-209.

2. Beyersmann D, Hartwig A. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch Toxicol 2008;82:493-512.

3. Schweikl H, Spagnuolo G, Schmalz G. Genetic and cellular toxicology of dental resin monomers. J Dent Res 2006;85:870-877.

4. Hossain S, Liu HN, Nguyen M, Shore G, Almazan G. Cadmium exposure induces mitochondria-dependent apoptosis in oligodendrocytes. Neurotoxicology 2009.

5. Matos de Souza R, Macedo de Menezes L. Nickel, chromium and iron levels in the saliva of patients with simulated fixed orthodontic appliances. Angle Orthod 2008;78:345-350.

6. Ribeiro LRS, D. M. F.; Marques, E. K. . Mutagênese ambiental. Canoas: Ulbra; 2003.

7. Holland N, Bolognesi C, Kirsch-Volders M, Bonassi S, Zeiger E, Knasmueller S et al. The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps. Mutat Res 2008;659:93-108.

8. Sarto F, Finotto S, Giacomelli L, Mazzotti D, Tomanin R, Levis AG. The micronucleus assay in exfoliated cells of the human buccal mucosa. Mutagenesis 1987;2:11-17.

9. Beserati NA. Immunoperoxidase detection of polycyclic aromatic hydrocarbon - DNA addutcts in mouth floor and buccal mucosa cells of smokers and nonsmokers. Environ Mol Mutagen. 2000;36:127-133.

10. Titenko-Holland N, Moore LE, Smith MT. Measurement and characterization of micronuclei in exfoliated human cells by fluorescence in situ hybridization with a centromeric probe. Mutat Res 1994;312:39-50.

11. Fenech M, Holland N, Chang WP, Zeiger E, Bonassi S. The HUman MicroNucleus Project--An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. Mutat Res 1999;428:271-283.

12. Thomas P, Holland N, Bolognesi C, Kirsch-Volders M, Bonassi S, Zeiger E et al. Buccal micronucleus cytome assay. Nat Protoc 2009;4:825-837.

13. Silva JE, B.; Henriques, J. A. P. . Genética toxicológica. Porto Alegre: Alcance; 2003.

14. Fenech M, Bolognesi C, Kirsch-Volders M, Bonassi S, Zeiger E, Knasmuller S et al. Harmonisation of the micronucleus assay in human buccal cells--a Human Micronucleus (HUMN) project (www.humn.org) initiative commencing in 2007. Mutagenesis 2007;22:3-4.

15. Wever DJ, Veldhuizen AG, Sanders MM, Schakenraad JM, van Horn JR. Cytotoxic, allergic and genotoxic activity of a nickel-titanium alloy. Biomaterials 1997;18:1115-1120.

16. Montanaro L, Cervellati M, Campoccia D, Prati C, Breschi L, Arciola CR. No genotoxicity of a new nickel-free stainless steel. Int J Artif Organs 2005;28:58-65.

17. Assad M, Chernyshov A, Leroux MA, Rivard CH. A new porous titanium-nickel alloy: part 2. Sensitization, irritation and acute systemic toxicity evaluation. Biomed Mater Eng 2002;12:339-346.

18. Assad M, Chernyshov A, Leroux MA, Rivard CH. A new porous titanium-nickel alloy: Part 1. Cytotoxicity and genotoxicity evaluation. Biomed Mater Eng 2002;12:225-237.

19. Westphalen GH, Menezes LM, Pra D, Garcia GG, Schmitt VM, Henriques JA et al. In vivo determination of genotoxicity induced by metals from orthodontic appliances using micronucleus and comet assays. Genet Mol Res 2008;7:1259-1266.

20. Faccioni F, Franceschetti P, Cerpelloni M, Fracasso ME. In vivo study on metal release from fixed orthodontic appliances and DNA damage in oral mucosa cells. Am J Orthod Dentofacial Orthop 2003;124:687-693; discussion 693-684.

21. Burgaz S, Demircigil GC, Yilmazer M, Ertas N, Kemaloglu Y, Burgaz Y. Assessment of cytogenetic damage in lymphocytes and in exfoliated nasal cells of dental laboratory technicians exposed to chromium, cobalt, and nickel. Mutat Res 2002;521:47-56.

22. Thomas P, NJ OC, Fenech M. Telomere length in white blood cells, buccal cells and brain tissue and its variation with ageing and Alzheimer's disease. Mech Ageing Dev 2008;129:183-190.

23. O'Callaghan N, Dhillon V, Thomas P, Fenech M. A quantitative real-time PCR method for absolute telomere length. Biotechniques 2008;44:807-809.

Table I - MN frequency comparison among each period

Periods	n	Average	SD	*p*
T1	10	3,00	3,09	0,284
T2	10	1,70	1,89	
T1	10	3,00	3,09	0,031
T3	10	1,30	1,06	
T2	10	1,70	1,89	0,522
T3	10	1,30	1,06	

Figure 1 - MN frequency for each period and patient

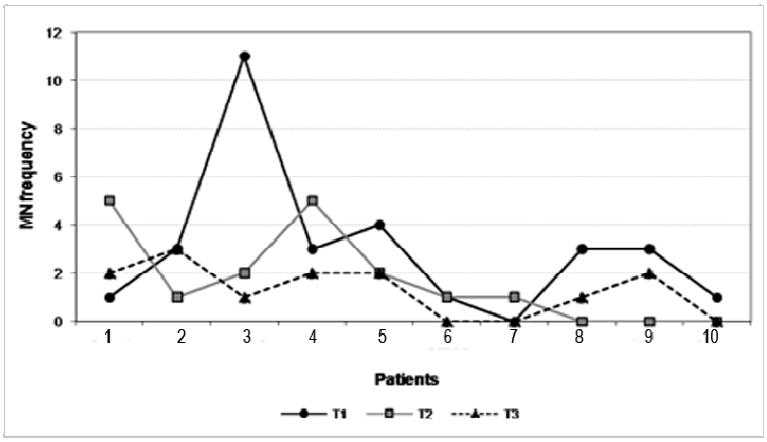
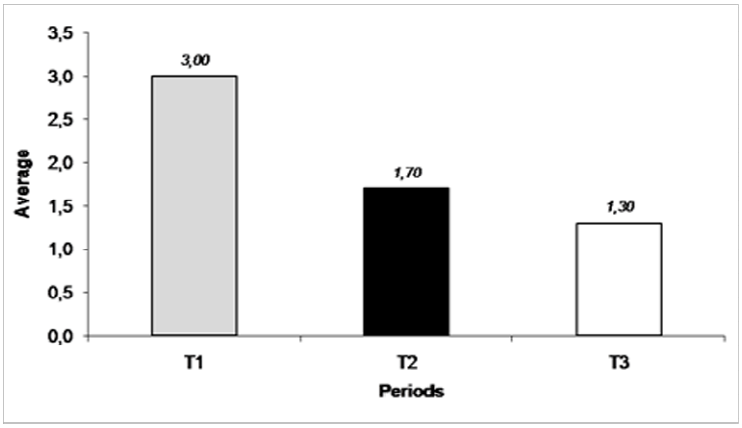


Figure 2 - Average MN frequency for each period



5 DISCUSSÃO GERAL

A importância do estudo da biocompatibilidade dos materiais odontológicos é um consenso na literatura[29-32], uma vez que o emprego de materiais biocompatíveis é essencial para garantir um tratamento biologicamente seguro aos pacientes[14]. A negligência dessa propriedade pode acarretar um aumento do risco à saúde dos pacientes, equipe odontológica e laboratorial[29].

A maioria dos metais utilizados na Ortodontia apresenta composição similar ao aço inoxidável (18% cromo e 8% níquel). Esses metais são potencialmente relacionados a efeitos citotóxicos, genotóxicos e carcinogênicos[4,6]. Determinados acessórios ortodônticos (anéis, bráquetes e máscaras faciais) contêm algum tipo de solda, cuja composição é comumente à base de prata e cobre, sendo que também apresentam outros metais como cádmio. A prata é considerada um metal tóxico para humanos[33], podendo alterar o metabolismo celular em exposições crônicas de baixa dosagem[22]. Da mesma forma, o cádmio apresenta efeitos tóxicos relevantes (nefrotoxicidade, neurotoxicidade) em diversos organismos[7,10,21,34], sendo também poluente e carcinógeno[7].

Além dos efeitos deletérios dos metais, outros constituintes de materiais ortodônticos apresentam efeitos adversos. A utilização de resina acrílica em aparelhos removíveis, contenção e acessórios fixos tem sido apontada como fator etiológico de reações de hipersensibilidade[9]. Efeitos citotóxicos *in vitro* também são atribuídos às resinas acrílicas, especialmente as quimicamente ativadas[35]. A presença de monômero não polimerizado, proveniente das resinas compostas utilizadas para colagem ortodôntica, é relacionada a efeitos de hipersensibilidade, citotoxicidade, genotoxicidade e alterações da reposta imune[36]. O uso do látex em diversos materiais odontológicos (luvas, lençol de isolamento e acessórios ortodônticos, como elásticos ortodônticos) é apontado com causador de reações alérgicas importantes[37-39]. Tendo em vista as evidências deletérias dos constituintes dos materiais ortodônticos, é muito importante a realização de estudos que investiguem os efeitos biológicos desses materiais.

A avaliação da biocompatibilidade dos materiais odontológicos compreende a abordagem de diferentes aspectos dos mesmos como: resistência a corrosão, potenciais citotóxicos, genotóxicos e carcinógenos. Deve-se considerar que a determinação da biocompatibilidade representa um processo complexo que envolve uma série de testes *in vitro* e *in vivo*[40]. Nesse estudo, a biocompatibilidade de materiais ortodônticos foi avaliada sob dois aspectos: 1) Citotoxicidade: por meio de testes *in vitro,* utilizando-se *Saccharomyces cerevisiae,* como organismo modelo 2) Genotoxicidade: optando-se pelo emprego dos experimentos: ensaio citoma bucal de micronúcleos, teste de micronúcleos e ensaio cometa em pacientes sob tratamento ortodôntico.

O emprego de *Saccharomyces cerevisiae* em testes de citotoxicidade é recomendado, devido às similaridades desse microorganismo com células humanas e animais, uma vez que o mesmo é eucarioto e unicelular. Além disso, muitas vantagens estão associadas à sua utilização como: fácil cultivo, manipulação e controle, obtenção de experimentos com grande quantidade amostral, rapidez e baixo custo[18,41,42]. Esse microorganismo é freqüentemente utilizado para avaliação da toxicidade de diferentes metais[43-46].

Dentre os testes de avaliação da genotoxicidade, o teste de micronúcleos identifica danos cromossômicos após a divisão celular, sendo a técnica mais utilizada para detecção de agentes clastogênicos (que quebram cromossomos) e aneugênicos (que induzem aneuploidia, ou segregação cromossômica anormal), sendo extremamente sensível para o monitoramento de danos genéticos[27]. Por sua vez, o ensaio citoma bucal de micronúcleos é um método minimamente invasivo, que provê a análise de danos do DNA, instabilidade cromossômica, morte celular e potencial regenerativo dos tecidos da mucosa bucal humana. É observado um aumento do uso desse método em estudos epidemiológicos moleculares para investigação do impacto de exposição à genotoxicinas, nutrição, estilo de vida e genótipo em danos do DNA, segregação cromossômica e morte celular[26,27].

Dentre os materiais ortodônticos utilizados nesse estudo, o único que apresentou citotoxicidade foi a solda de prata (Artigo 1), que mostrou uma redução significativa da viabilidade celular de *S. Cerevisiae* em ambas exposições, direta e

indireta (saliva artificial), a esse microorganismo. Esse achado enfatiza a ação tóxica desse material demonstrada previamente por vários estudos[10,47-49]. Dessa forma, evidencia-se a necessidade do desenvolvimento de materiais mais biocompatíveis para substituição da solda de prata. Nesse sentido, a solda a laser apresenta-se como uma alternativa promissora, uma vez que esse material mostrou, em estudos anteriores, uma redução marcante da toxicidade celular em relação à solda de prata[47-49], apresentando uma tolerância biológica celular adequada[48]. Apesar dos materiais (bráquetes, fios, resinas compostas e elastômeros) não mostrarem efeitos citotóxicos nesse estudo, seus efeitos biológicos previamente relatados (hipersensibilidade, citotoxicidade e genotoxicidade) não devem ser negligenciados e constantemente estudados.

Em relação à genotoxicidade, este estudo mostrou que o uso dos aparelhos tipo Haas e Hyrax e Ortodônticos fixos (liga de aço inoxidável) não demonstraram aumento dos biomarcadores de: danos no DNA (freqüência de micronúcleos e nuclear *BUD),* morte celular (cromatina condensada, cariorexe, cariólise e picnose) e proliferação celular (núcleo duplo) indicados pelo ensaio citoma bucal de micronúcleos (Artigo 2). Da mesma forma, os aparelhos expansores maxilares (Hyrax e Haas) não mostraram potencial genotóxico (aumento da freqüência de micronúcleos após 30 dias do uso dos aparelhos) em grupo de pacientes ortodônticos jovens (Artigo 3).

Os achados indicativos de ausência de genotoxicidade associados ao uso de aparelhos ortodônticos encontrados nesse estudo vão ao encontro de resultados de muitos estudos *in vitro*[12-15]*.* De acordo com Westphalen et al. (2008)[50], a genotoxicidade de aparelhos ortodônticos fixos (liga aço inoxidável) não pode ser totalmente evidenciada, uma vez que se observaram resultados contrastantes como, aumento da freqüência de micronúcleos e diminuição de danos induzidos pelo teste cometa, 30 e 10 dias, respectivamente, após a colocação de aparelho, em um grupo de pacientes. Entretanto, Faccioni et al. (2003)[3] demonstraram que metais (níquel e cobalto), quando liberados dos aparelhos ortodônticos fixos, podem causar quebras no DNA de células da mucosa bucal de pacientes ortodônticos. Da mesma forma, a exposição aos metais (cromo, cobalto e níquel) mostrou um aumento da freqüência

de micronúcleos tanto em células nasais quanto em linfócitos periféricos de técnicos dentários de laboratórios[51]. Frente a essas evidências, acredita-se que a investigação da genotoxicidade dos aparelhos ortodônticos não esteja complementarmente esgotada.

O entendimento das correlações celulares é extremamente importante uma vez que as mesmas são indicativas de associação biológica entre as células e seu desenvolvimento seqüencial[52], entretanto ainda é pouco abordado. Esse estudo (Artigo 2) foi um dos primeiros que avaliaram a dinâmica das células bucais, e observou-se a existência de correlações positivas e negativas de biomarcadores celulares detectados pelo uso do ensaio citoma bucal de micronúcleos. Essas evidências fornecem dados iniciais para o entendimento das correlações celulares, entretanto, acredita-se que mais estudos devam ser realizados para melhor compreender o processo complexo da dinâmica celular.

6 REFERÊNCIAS

1.Noort RV, Introdução aos materiais dentários. São Paulo: Artmed; 2004.

2.Costa CAS, Souza PPC. Testes de citotoxicidade em cultura de células. São Paulo: Artmed; 2005.

3.Faccioni F, Franceschetti P, Cerpelloni M, Fracasso ME. In vivo study on metal release from fixed orthodontic appliances and DNA damage in oral mucosa cells. Am J Orthod Dentofacial Orthop. 2003 124(6):687-93.

4.Valko M, Morris H, Cronin MT. Metals, toxicity and oxidative stress. Curr Med Chem. 2005 12(10):1161-208.

5.Sethi PK, Khandelwal D. Cadmium exposure: health hazards of silver cottage industry in developing countries. J Med Toxicol. 2006 2(1):14-5.

6.Beyersmann D, Hartwig, A. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch Toxicol. 2008 82(8): 493-512.

7.Hossain S, Liu HN, Nguyen M, Shore G, Almazan G. Cadmium exposure induces mitochondria-dependent apoptosis in oligodendrocytes. Neurotoxicology. 2009 30(4):544-54.

8.Schweikl H, Spagnuolo G, Schmalz G. Genetic and cellular toxicology of dental resin monomers. J Dent Res. 2006 85(10):870-7.

9.Goncalves TS, Morganti MA,Campos LC,Rizzatto SM, Menezes LM. Allergy to auto-polymerized acrylic resin in an orthodontic patient. Am J Orthod Dentofacial Orthop. 2006 129(3):431-5.

10.Freitas MP, Oshima HM, Menezes LM, Machado DC, Viezzer C. Cytotoxicity of silver solder employed in orthodontics. Angle Orthod. 2009 79(5): 939-44.

11.Menezes LM, Quintao CA, Bolognese AM. Urinary excretion levels of nickel in orthodontic patients. Am J Orthod Dentofacial Orthop. 2007 131(5):635-8.

12.Assad M, Chernyshov A, Leroux MA, Rivard CH. A new porous titanium-nickel alloy: Part 1. Cytotoxicity and genotoxicity evaluation. Biomed Mater Eng. 2002 12(3):225-37.

13.Assad M, Chernyshov A, Leroux MA, Rivard CH. A new porous titanium-nickel alloy: part 2. Sensitization, irritation and acute systemic toxicity evaluation. Biomed Mater Eng. 2002 12(4):339-46.

14.Montanaro L, Cervellati M, Campoccia D, Prati C,Breschi L, Arciola CR. No genotoxicity of a new nickel-free stainless steel. Int J Artif Organs. 2005 28(1):58-65.

15.Wever DJ, Veldhuizen AG, Sanders MM, Schakenraad JM, Van Horn JR. Cytotoxic, allergic and genotoxic activity of a nickel-titanium alloy. Biomaterials. 1997 18(16):1115-20.

16.Grimsdottir MR, Hensten-Pettersen A, Kullmann A. Cytotoxic effect of orthodontic appliances. Eur J Orthod. 1992 14(1):47-53.

17.Mockers O, Deroze D, Camps J. Cytotoxicity of orthodontic bands, brackets and archwires in vitro. Dent Mater. 2002. 18(4): p. 311-7.

18.De Freitas J, Wintz H, Kim JH, Poynton H, Fox T,Vulpe C. Yeast, a model organism for iron and copper metabolism studies. Biometals. 2003 16(1):185-97.

19.Kao CT, Ding SJ, Min Y, Hsu TC, Chou MY,Huang TH. The cytotoxicity of orthodontic metal bracket immersion media. Eur J Orthod. 2007 29(2):198-203.

20.Lewis JB, Wataha JC, Randol TM, McCloud VV, Lockwood PE.Metal ions alter lipopolysaccharide-induced NF kappa B binding in monocytes. J Biomed Mater Res A. 2003 67(3):868-75.

21.Watjen W, Haase H, Biagioli M, Beyersmann D. Induction of apoptosis in mammalian cells by cadmium and zinc. Environ Health Perspect. 2002 110 (Suppl 5):865-7.

22.Wataha JC, Lockwood PE, Schedle A, Noda M, Bouillaguet S. Ag, Cu, Hg and Ni ions alter the metabolism of human monocytes during extended low-dose exposures. J Oral Rehabil. 2002 29(2):133-9.

23.Issa Y, Brunton P, Waters CM, Watts DC. Cytotoxicity of metal ions to human oligodendroglial cells and human gingival fibroblasts assessed by mitochondrial dehydrogenase activity. Dent Mater. 2008 24(2):281-7.

24.Bohrer PL. Avaliação das alterações citopatológicas da mucosa bucal clinicamente normal exposta a carcinógenos (dissertação). Porto Alegre (RS): Universidade Federal do Rio Grande do Sul; 2003.

25.Fenech M, Holland N, Chang WP, Zeiger E, Bonassi S. The HUman MicroNucleus Project--An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. Mutat Res. 1999 428(1-2):271-83.

26.Thomas P, Holland N, Bolognesi C, Kirsch-Volders M, Bonassi S, Zeiger E.Buccal micronucleus cytome assay. Nat Protoc. 2009 4(6):825-37.

27.Holland N, Bolognesi C, Kirsch-Volders M, Bonassi S, Zeiger E, Knasmueller S, et al. The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps. Mutat Res. 2008 659(1-2):93-108.

28.Fenech M., Holland N,Knasmueller S, Burgaz S, Bonassi S. Report on the buccal micronucleus assay workshop organized by the International Human Micronucleus (HUMN) project--Antalya, Turkey 2007. Mutagenesis. 2009 24(2): 199-201.

29.Wataha JC. Principles of biocompatibility for dental practitioners. J Prosthet Dent. 2001 86(2): 203-9.

30.Eliades T. Orthodontic materials research and applications: part 2. Current status and projected future developments in materials and biocompatibility. Am J Orthod Dentofacial Orthop. 2007, 131(2):253-62.

31.Murray PE, Garcia-Godoy F. Comparison of the clinical and preclinical biocompatibility testing of dental materials: are the ISO usage tests meaningful? J Biomed Mater Res A. 2007 81(1):51-8.

32.St John KR. Biocompatibility of dental materials. Dent Clin North Am. 2007 51(3):747-60.

33.Pelkonen KH, Heinonen-Tanski H, Hanninen OO. Accumulation of silver from drinking water into cerebellum and musculus soleus in mice. Toxicology. 2003 186(1-2):151-7.

34.Watjen W, Cox M, Biagioli M, Beyersmann D. Cadmium-induced apoptosis in C6 glioma cells: mediation by caspase 9-activation. Biometals. 2002 15(1): p. 15-25.

35.Rose EC, Bumann J, Jonas IE, Kappert HF. Contribution to the biological assessment of orthodontic acrylic materials. Measurement of their residual monomer output and cytotoxicity. J Orofac Orthop. 2000 61(4):246-57.

36.Brackett MG, Bouillaguet S, Lockwood PE, Rotenberg S, Lewis JB, Messer RL, et al. In vitro cytotoxicity of dental composites based on new and traditional polymerization chemistries. J Biomed Mater Res B Appl Biomater. 2007 81(2): 397-402.

37.Burke FJ, Wilson NH. Allergic reactions to rubber gloves in dental patients. Br Dent J. 1992 173(4): 124.

38.Levy DA, Charpin D, Pecquet C, Leynadier F, Vervloet D. Allergy to latex. Allergy. 1992 47(6):579-87.

39.Conde-Salazar L, del-Rio E, Guimaraens D, Gonzalez Domingo A. Type IV allergy to rubber additives: a 10-year study of 686 cases. J Am Acad Dermatol. 1993 29(21):176-80.

40.Wataha JC. Biocompatibility of dental casting alloys: a review. J Prosthet Dent. 2000 83(2): 223-34.

41.Perego P, Howell SB. Molecular mechanisms controlling sensitivity to toxic metal ions in yeast. Toxicol Appl Pharmacol. 1997 147(2):312-8.

42.Avery SV, Metal toxicity in yeasts and the role of oxidative stress. Adv Appl Microbiol. 2001 49:111-42.

43.Soares EV, Hebbelinck K, Soares, HM.Toxic effects caused by heavy metals in the yeast Saccharomyces cerevisiae: a comparative study. Can J Microbiol. 2003 49(5): p. 336-43.

44.Viau C, Pungartnik C, Schmitt MC, Basso TS,Henriques JÁ, Brendel M. Sensitivity to Sn2+ of the yeast Saccharomyces cerevisiae depends on general energy metabolism, metal transport, anti-oxidative defences, and DNA repair. Biometals. 2006 19(6):705-14.

45.Chen C, Wang J. Response of Saccharomyces cerevisiae to lead ion stress. Appl Microbiol Biotechnol. 2007 74(3):683-7.

46.Liang Q, Zhou B. Copper and manganese induce yeast apoptosis via different pathways. Mol Biol Cell. 2007 18(12):4741-9.

47.Vande Vannet B, Hanssens JL, Wehrbein H. The use of three-dimensional oral mucosa cell cultures to assess the toxicity of soldered and welded wires. Eur J Orthod. 2007 29(1):60-6.

48.Sestini S, Notarantonio L, Cerboni B, Alessandrini C, Fimiani M, Nannelli P, et al. In vitro toxicity evaluation of silver soldering, electrical resistance, and laser welding of orthodontic wires. Eur J Orthod. 2006 28(6): p. 567-72.

49.Solmi R, Martini D, Zanarini M, Isaza Penco S, Rimondini L, Carinci P, et al. Interactions of fibroblasts with soldered and laser-welded joints. Biomaterials. 2004 25(4):735-40.

50.Westphalen GH, Menezes LM, Pra D, Garcia GG, Schmitt VM, Henriques JA, *et al*. In vivo determination of genotoxicity induced by metals from orthodontic appliances using micronucleus and comet assays. *Genet Mol Res.* 2008 7*:*1259-66.

51.Burgaz S, Demircigil GC, Yilmazer M, Ertas N, Kemaloglu Y, Burgaz Y. Assessment of cytogenetic damage in lymphocytes and in exfoliated nasal cells of dental laboratory technicians exposed to chromium, cobalt, and nickel. Mutat Res. 2002 521(1-2):47-56.

52. Thomas P, et al., The buccal cytome and micronucleus frequency is substantially altered in Down's syndrome and normal ageing compared to young healthy controls. Mutat Res. 2008 638(1-2): p. 37-47.

7 ANEXOS

ANEXO A - Comprovante de submissão do Artigo 1

Gmail by Google

Graziela Henriques Westphalen <grazielaorto@gmail.com>

Submission Confirmation for your paper

1 mensagem

Dental Materials <dentistry.dentmatj@manchester.ac.uk> **5 de novembro de 2009 21:41**
Para: grazielaorto@gmail.com

Dear Mrs Graziela Westphalen,

Your submission entitled "Cytotoxicity of orthodontic materials assessed by survival tests in Saccharomyces cerevisiae" has been received by Dental Materials

You may check on the progress of your paper by logging on to the Elsevier Editorial System as an author. The URL is http://ees.elsevier.com/dema/. Your username is Your username is: Grazihw and your password is Your password is: westphalen377.

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Thank you for submitting your work to this journal.

Kind regards,

Elsevier Editorial System
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ANEXO B - Comprovante de submissão do Artigo 2

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Graziela Henriques Westphalen <grazielaorto@gmail.com>

09-0688 Associate Editor Assigned

1 mensagem

nhayden@iadr.org <nhayden@iadr.org> **4 de novembro de 2009 12:48**
Responder a: nhayden@iadr.org
Para: grazielaorto@gmail.com

Dear Dr. Westphalen,

On November 4, 2009, I received your manuscript entitled "Changes in Basal Cell Frequency in Orthodontic Patients."

Your paper has been assigned MS# 09-0688.

You may check on the status of your manuscript while it is in review by selecting the "Check Manuscript Status" link under the following URL:

http://jdr.msubmit.net/cgi-bin/main.plex?el=A2N7Dxg1A1KSo3F2A9arOzv698x3uGMaoorjkK6QZ

If you have questions, you can also get help by e-mailing nhayden@iadr.org.

Thank you for submitting your work to Journal of Dental Research.

Sincerely,

Norm Hayden
Publications Coordinator
IADR/AADR
nhayden@iadr.org

ANEXO C - Comprovante de submissão do Artigo 3

THE ANGLE ORTHODONTIST
ONLINE MANUSCRIPT SUBMISSION AND PEER REVIEW

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Detailed Status Information

Manuscript #	110809-629
Current Revision #	0
Submission Date	2009-11-08 04:20:17
Current Stage	Initial QC Started
Title	In vivo genotoxicity assessment of maxillary expander appliances
Running Title	Genotoxicity of maxillary expander appliances
Manuscript Type	Original Article
Special Section	N/A
Corresponding Author	Graziela Westphalen (Pontifícia Universidade Católica do Rio Grande do Sul)
Contributing Authors	Luciane Menezes , Fabiane Azeredo , Gabriela Schimtt , Daniela Pra , João Antônio Henriques , Renata Medina
Financial Disclosure	I certify that all financial and material support for this research and work are clearly identified in the manuscript. Details regarding this support have been fully outlined in my cover letter.
Abstract	Some components of maxillary expansor appliances (Haas and Hyrax) might induce genotoxic effects. The aim of this study was to assess the genotoxicity related to wearing maxillary expansor appliances (Haas and Hyrax) in group of patients. Micronucleus assays were carried out in buccal cells, which were sampled before (T1), 10 (T2) and 30 (T3) days after the placement of maxillary expansor appliances. The frequency of micronuclei was compared among three periods, using the Wilcoxon test ($p<0.05$). Results showed that compared with T1, the micronucleus frequency decreased significantly at T3. Therefore, the appliances did not show any genotoxic effects in these patients. However, further studies in long-term models using different methodologies are necessary to better understand the biological effects.
Key Words	Orthodontics, genotoxicity, micronucleus test
Conflict of Interest	**I have no conflict of interest that I should disclose.**

Stage	Start Date	End Date	Approximate Duration
Initial QC Started	2009-11-08 04:42:59		
Author Approved Converted Files	2009-11-08 04:42:58		

ANEXO D – Artigo publicado durante o Doutorado

Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

In vivo determination of genotoxicity induced by metals from orthodontic appliances using micronucleus and comet assays

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ABSTRACT. Orthodontic appliances are usually made of stainless steel, which contains metals such as nickel, chromium and iron that have been associated with DNA damage. The aim of the present study was to determine the genetic toxicity associated with orthodontic fixed appliances in twenty healthy patients (16 ± 2.5 years) undergoing orthodontic treatment (fixed appliances - basic composition: stainless steel alloy), using the micronucleus (MN) and comet (CA) assays in buccal cells. Primary DNA damage level, as assessed by the CA, was low either before the beginning (1.5 ± 1.05 damage index - DI) or 10 days after the placement of the orthodontic appliance (2.5 ± 3.08 DI) and did not change significantly between these time points (P = 0.0913). Conversely, there was a significant increase in MN frequency 30 days after the beginning of the treatment (P = 0.0236). In this study, the MN assay was shown to be more sensitive than the CA. Other investigations are necessary in order to assess the genotoxic potential of orthodontic fixed appliances associated with long-term studies concerning these effects in orthodontic patients.

Key words: Genotoxicity; Mutagenic tests; Metals; Dental materials; Orthodontics

INTRODUCTION

Orthodontic appliances are usually made of stainless steel alloy, which contains metals such as chromium, nickel and iron (Staerkjaer and Menné, 1990; Bass et al., 1993). The mouth properties (thermal, microbiological and enzymatic) offer an ideal environment for the biodegradation of orthodontic appliances (Faccioni et al., 2003; Thomas et al., 2007, 2008; Amini et al., 2008; Matos de Souza and Macedo de Menezes, 2008), consequently facilitating the release of metal ions that are related to adverse health effects, such as cellular and genetic toxicity (Munksgaard, 1992; Wataha, 2000; Dayan and Paine, 2001; Valko et al., 2005; Thomas et al., 2007, 2008).

Genotoxicity comprises either mutagenic or carcinogenic processes. Thus, the genotoxic properties of metals from orthodontic appliances are defined as an essential criterion to select these materials in a safe biological manner for patients (Montanaro et al., 2005).

The assessment of genotoxic agents can be performed through the application of some well-established endpoints such as the micronucleus (MN) frequency, as determined by the MN assay, or primary DNA damage, as accessed by the comet assay (CA). The combination of the two assays is considered to be very beneficial, because they show supplementary characteristics (Van Goethem et al., 1997). The MN assay is based on the frequency of MN, structures that originate from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division (Fenech et al., 1999). Thus, MN may arise from either DNA breakage leading to acentric chromosome fragments or from chromosome/chromatin lagging in anaphase. The formation of MN is considered to be an effective biomarker of diseases and processes associated with the induction of DNA damage. Another assay that has been indicated in order to complement the MN result is the alkaline single cell gel electrophoresis assay (Van Goethem et al., 1997), the CA, which measures single- and/or double-strand breaks in a cell by the cell approach. The CA is considered a quick, simple, sensitive, reliable, and fairly inexpensive way of measuring DNA damage (Collins et al., 1997).

The aim of the present research was to determine the genotoxicity induced by metals from orthodontic appliances, by employing both the MN and the CA in a group of healthy patients undergoing orthodontic treatment.

MATERIAL AND METHODS

Patients and treatment

Twenty healthy patients (14 females) with an average age of 16 ± 2.5 years, undergoing orthodontic treatment, were enrolled in this study. Orthodontic appliances were made of stainless steel (0.07% carbon, 1.0% manganese, 1.0% silicon, 15.5-17.5% chromium, 3-5% nickel, 3-5% copper, 0.15-0.45% niobium + tantalum) in both arches, consisting basically of an average of 20 bonded brackets and four bands (3M Unitek®, Monrovia, CA, USA). Smoking or drinking or illnesses related to any genetic damage increase were not reported by any patient. The patients' consent was obtained after a full explanation of the objective of the study. The research was approved by the University's Ethics Committee (Pontifícia Universidade do Rio Grande do Sul, Brazil).

Buccal cell sampling

The samples were collected before (control) and after the placement of the orthodontic appliances. For the CA, the samples were obtained before and 10 days after the placement of the orthodontic appliances. For the MN assay, cells were sampled before and 30 days after the placement of the orthodontic appliances. Buccal cells were collected from each individual by gentle brushing of the inside part of the lower lip with a cytological brush, after washing out the mouth several times with tepid distilled water to remove exfoliated dead cells. The brushes were stirred in 50-mL plastic tubes containing 20 mL phosphate-buffered saline (PBS). Cells were washed twice, with centrifugation at 1500 rpm for 10 min at room temperature, and resuspended in PBS, which was employed for the CA or MN assay.

Comet assay

The alkaline version of the CA was employed in this study (Speit and Hartmann, 1999; Faccioni et al., 2003). Briefly, 10 µL cell suspension was mixed with 75 µL low-melting-point agarose (0.7%) and added to a slide precoated with 100 µL agarose (1%). Lysis was performed overnight at pH 10. Cells were then placed in a electrophoresis chamber, exposed to alkali, pH 13, for 25 min, and electrophoresis was performed for 20 min at 25 V (0.86 V/cm) and 300 mA, at room temperature. The slides were neutralized, fixed, and stained with silver nitrate (Nadin et al., 2001). The slides were examined under a light microscope (Axiolab, Zeiss) at 1000X magnification. Fifty randomly selected cells of each subject (25 cells for each of two replicate slides) were visually scored according to five classes, based on tail size (from undamaged - 0, to maximally damaged - 4). Damage index (DI) was thus assigned to each individual, according to the sum of the classes attributed to each cell, ranging from 0 (completely undamaged: 50 cells x 0) to 200 (with maximum damage: 50 cells x 4) (Hartmann et al., 2003). The DI is based on the length of migration and on the amount of DNA in the tail and is considered to be a sensitive measure of DNA. International guidelines and recommendations for the CA consider that visual scoring of comets is a well-validated evaluation method as it is highly correlated with computer-based image analysis (Burlinson et al., 2007).

Micronucleus assay

Buccal cells were collected and analyzed according to a standard protocol described elsewhere by Titenko-Holland et al. (1994). Slides of buccal cells were prepared by dropping the washed cell suspension onto pre-warmed slides (37°C). After dropping, the cells were allowed to air-dry and fixed in methanol (80%, v/v) at 0°C for 20 min. Staining was performed with May-Grunwald-Giemsa according to a standard protocol (Titenko-Holland et al., 1994). Only cells that were not smeared, clumped or overlapping and that contained intact nuclei were included in the analysis. MN were identified according to the following characteristics: i) less than 1/3 diameter of the main nucleus; ii) the same plane of focus; iii) the same color, texture and refraction as the main nucleus; iv) smooth oval or round shape, and v) clearly separated from the main nucleus (Titenko-Holland et al., 1994). Cells were observed in oil immersion at 1000X magnification with a light microscope (Axiolab, Zeiss) to determine the presence of MN cells, as established by Sarto et al., 1987.

Statistical analysis

The one-tailed *t*-test with Welch's correction was used to compare DI obtained by the CA, and the one-tailed Fisher exact test was used to compare the number of patients with MN before and after the placement of orthodontic appliance.

RESULTS

Primary DNA damage level, as assessed by the CA, was low either before the beginning (1.5 ± 1.05) or 10 days after the placement of orthodontic appliance (2.5 ± 3.08) and did not change significantly between these time points (P = 0.0913). Most cells were classified as class 0 regarding DNA damage extent, as depicted in Figure 1.

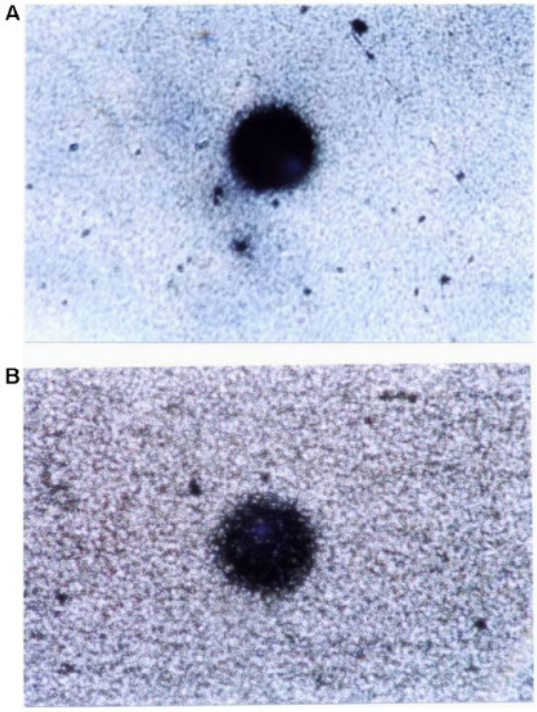

Figure 1. The comet assay. Nuclei of oral epithelium cells (1000X magnification) from a male patient, 11 years old, classified as zero class, before (A) and 10 days after (B) the placement of the orthodontic appliance.

Conversely, there was a significant increase in MN frequency (P = 0.0213) 30 days after the placement of orthodontic appliances (Table 1). Figure 2 illustrates an MN cell at 30 days after the placement of the orthodontic appliance.

Table 1. Comparison of mononuclear (MN) cell frequency in the periods before and 30 days after the placement of orthodontic appliances.

MN cells/1000 cells	Before		After 30 dias	
	Number of cases	%	Number of cases	%
0	20	100	15	75
1	0	0	1	5
2	0	0	2	10
3	0	0	2	10
Total	20	100	20	100

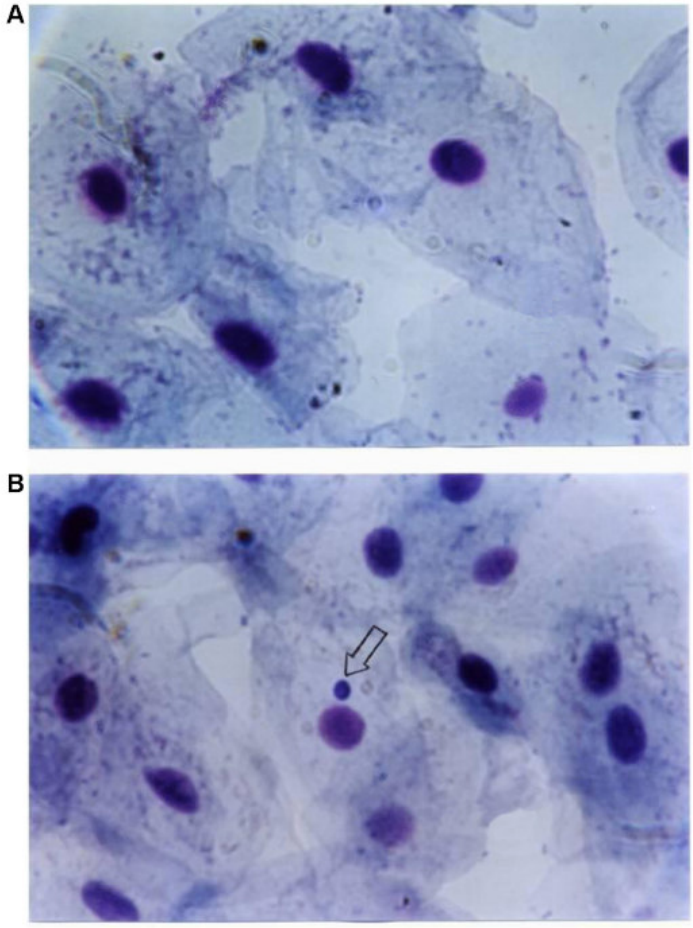

Figure 2. The micronucleus test. Cells of the oral epithelium (1000X magnification) from a female patient, 20 years old. **A.** Before and **B.** After 30 days of placement of orthodontic appliances. One micronucleus is indicated (arrow) in a binucleated cell in B.

DISCUSSION

When orthodontic appliances are present in the oral cavity they are usually subject to corrosion processes, which lead to the release of metals (Ağaoğlu et al., 2001; Levrini et al., 2006; Matos de Souza and Macedo de Menezes, 2008). Some metallic elements present in orthodontic appliances, such as nickel and chromium, are known to be potential carcinogenic and mutagenic agents (Currents Conferences, 1984; Oller et al., 1997; Novelli et al., 1998; Wataha, 2000; Dayan and Paine, 2001; Burgaz et al., 2002; Costa, 2002; Zoroddu et al., 2002). The amount of metals released from orthodontic appliances in saliva or blood samples was significantly below the average dietary intake and did not reach toxic concentrations (Kocadereli et al., 2000; Ağaoğlu et al., 2001). However, it cannot be excluded that even nontoxic concentrations could be sufficient to induce biological effects in cells from the oral mucosa (Faccioni et al., 2003).

Current *in vivo* studies are aimed at representing the real condition of the oral cavity by sampling buccal cells, which are directly exposed to the appliances (Faccioni et al., 2003). Besides, there are some advantages of using this cell type: it is the least invasive method available for measuring DNA damage, and these cells could represent a preferred target site for early genotoxic events induced by carcinogenic agents entering the body via inhalation and ingestion (Jones et al., 1994; Holland et al., 2008). A cytobrush was used for sampling, which appears to be the most effective technique for collecting large numbers of oral mucosa cells (Holland et al., 2008).

Genotoxicity effects from orthodontic appliances were assessed by carrying out both MN and CA assays. This combination is recommended, since the CA could detect primary DNA damage (reparable) in a short period of time, while the MN assay detects chromosomal damage in a further stage (Van Goethem et al., 1997; Vrzoc and Petras, 1997). Therefore, the time of sampling was 10 days after the placement of the orthodontic appliance for the CA and 20 days later (30 days from the beginning of the exposure) for MN assay. The sampling time was suitable for a trial involving volunteers, and standard protocols were applied for both assays (Titenko-Holland et al., 1994; Speit and Hartmann, 1999). Nonetheless, since the viability of the buccal cells was not accessed in the present study, the results of the CA should be interpreted with caution.

CA has been used successfully to monitor DNA damage in human populations (Collins et al., 1997; Speit and Hartmann, 1999), and thus, it had a relevant application in this research. Moreover, it is a quick, simple, sensitive, reliable, and fairly inexpensive way of measuring DNA damage (Collins et al., 1997). In this research, the CA results reveal that orthodontic appliances did not induce any genetic damage. Similar findings were observed when CA was carried out in cultured human gingival keratinocytes exposed for up to 14 days to orthodontic brackets (Tomakidi et al., 2000). On the other hand, when the CA was applied in an *in vivo* study (55 orthodontic patients and 30 control subjects), it was demonstrated that metallic ions such as nickel and cobalt released from orthodontic appliances could induce DNA damage in oral mucosa cells (Faccioni et al., 2003). This difference may be explained by the fact that this previous study was a cross-sectional study associated with a larger number of patients analyzed using the appliances for a much longer period (2-3 years).

The MN assay has been applied in biological monitoring of human populations exposed to a variety of mutagenic and carcinogenic chemical or physical agents (Holland et al., 2008). The MN assay showed an increase in MN cells 30 days after the placement of the orthodontic appliances. A similar increase has been observed in epithelial cells exposed to some metals (Benova et

al., 2002; Burgaz et al., 2002; Danadevi et al., 2004; Lewiska, 2007). However, no trials have been reported verifying genotoxicity induced by metals from orthodontic appliances.

Despite the MN increase observed, orthodontic materials have not exhibited cytotoxicity or genotoxicity in several trials (Wever et al., 1997; Assad et al., 2002a,b; Montanaro et al., 2005).

The difference in results between the two assays usually occurs due to specific genetic endpoints that each of them is able to elicit. Generally, the CA detects more DNA damage than the MN assay. Positive results in the CA do not always correspond to positive results in the MN assay, especially when the exposure to genotoxic agents is small (Van Goethem et al., 1997; Martino-Roth et al., 2003; Silva et al., 2003; Ribeiro et al., 2003). Nevertheless, in this study the MN assay was shown to be more sensitive than the CA. Furthermore, the assays were conducted at different times and a smaller number of cell samples were analyzed in the CA.

The divergence of results found in this study, the little research in this field and the importance of this subject for the health status of orthodontic patients are reasons for further studies with larger samples and long-term follow-up analysis. These strategies will yield valuable data in order to better understand the genotoxic potential of metals from orthodontic devices, as well as to access the biological risk to which orthodontic patients may be submitted.

REFERENCES

Ağaoğlu G, Arun T, Izgi B and Yarat A (2001). Nickel and chromium levels in the saliva and serum of patients with fixed orthodontic appliances. *Angle Orthod.* 71: 375-379.

Amini F, Borzabadi FA, Jafari A and Rabbani M (2008). *In vivo* study of metal content of oral mucosa cells in patients with and without fixed orthodontic appliances. *Orthod. Craniofac. Res.* 11: 51-56.

Assad M, Chernyshov A, Leroux MA and Rivard CH (2002a). A new porous titanium-nickel alloy: part 1. Cytotoxicity and genotoxicity evaluation. *Biomed. Mater. Eng.* 12: 225-237.

Assad M, Chernyshov A, Leroux MA and Rivard CH (2002b). A new porous titanium-nickel alloy: part 2. Sensitization, irritation and acute systemic toxicity evaluation. *Biomed. Mater. Eng.* 12: 339-346.

Bass JK, Fine H and Cisneros GJ (1993). Nickel hypersensitivity in the orthodontic patient. *Am. J. Orthod. Dentofacial Orthop.* 103: 280-285.

Benova D, Hadjidekova V, Hristova R, Nikolova T, et al. (2002). Cytogenetic effects of hexavalent chromium in Bulgarian chromium platers. *Mutat. Res.* 514: 29-38.

Burgaz S, Demircigil GC, Yilmazer M, Ertas N, et al. (2002). Assessment of cytogenetic damage in lymphocytes and in exfoliated nasal cells of dental laboratory technicians exposed to chromium, cobalt, and nickel. *Mutat. Res.* 521: 47-56.

Burlinson B, Tice RR, Speit G, Agurell E, et al. (2007). Fourth international workgroup on genotoxicity testing: results of the *in vivo* comet assay workgroup. *Mutat. Res.* 627: 31-35.

Collins AR, Dobson VL, Dusinská M, Kennedy G, et al. (1997). The comet assay: what can it really tell us? *Mutat. Res.* 375: 183-193.

Costa M (2002). Molecular mechanisms of nickel carcinogenesis. *Biol. Chem.* 383: 961-967.

Currents Conferences (1984). Workshop: biocompatibility of metals in dentistry. *J. Am. Dent. Assoc.* 109: 469-471.

Danadevi K, Rozati R, Banu BS and Grover P (2004). Genotoxic evaluation of welders occupationally exposed to chromium and nickel using the Comet and micronucleus assays. *Mutagenesis* 19: 35-41.

Dayan AD and Paine AJ (2001). Mechanisms of chromium toxicity, carcinogenicity and allergenicity: review of the literature from 1985 to 2000. *Hum. Exp. Toxicol.* 20: 439-451.

Faccioni F, Franceschetti P, Cerpelloni M and Fracasso ME (2003). *In vivo* study on metal release from fixed orthodontic appliances and DNA damage in oral mucosa cells. *Am. J. Orthod. Dentofacial Orthop.* 124: 687-693.

Fenech M, Holland N, Chang WP, Zeiger E, et al. (1999). The human microNucleus project - An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. *Mutat. Res.* 428: 271-283.

Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, et al. (2003). Recommendations for conducting the *in vivo* alkaline Comet assay. 4th International Comet Assay Workshop. *Mutagenesis* 18: 45-51.

Holland N, Bolognesi C, Kirsch-Volders M, Bonassi S, et al. (2008). The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps. *Mutat.*

ANEXO E – Certificado de apresentação dos resultados da pesquisas em evento internacional

IV Meeting in Fundamental Aspects of DNA Repair and Mutagenesis

November 8 - 10, 2009 | Belo Horizonte | Minas Gerais

CERTIFICADO

Certificamos que o trabalho intitulado **Saccharomyces cerevisiae cell viability and buccal mucosa basal cell frequency are significantly reduced by orthodontic appliances** de autoria de **Graziela Henriques Westphalen** e colaboradores foi apresentado no IV Meeting in Fundamental Aspects of DNA Repair and Mutagenesis, no período de 08 a 10 de novembro, no Auditório da Reitoria da Universidade Federal de Minas Gerais.

Belo Horizonte, 10 de novembro de 2009

Carlos Renato Machado
Coordenador

Elza T. Sakamoto Hojo
Presidente SBMCTA

Bioeasy Biomolecular

SBMCTA
SOCIEDADE BRASILEIRA DE MUTAGÊNESE CARCINOGÊNESE E TERATOGÊNESE AMBIENTAL

20 anos SBMCTA

ANEXO F – Ata de pré-defesa de tese, com aprovação do projeto de pesquisa

Pontifícia Universidade Católica do Rio Grande do Sul
FACULDADE DE ODONTOLOGIA
PÓS-GRADUAÇÃO

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA
ÁREA DE CONCENTRAÇÃO: MATERIAIS DENTÁRIOS
NÍVEL: DOUTORADO

ATA DE PRÉ-DEFESA DE TESE

Data: 30/04/2008

Candidata: **GRAZIELA HENRIQUES WESTPHALEN**

Orientador(a): Profa. Dra. Luciane Macedo de Menezes

Título da pesquisa: Toxicidade celular e genética dos materiais ortodônticos

Comissão Avaliadora: Profa. Dra. Maria Ivete Bolzan Rockenbach
Prof. Dr. Telmo Bandeira Berthold

Parecer:
APROVADO, SUGERINDO-SE PEQUENAS CORREÇÕES E SUGESTÕES

Ass.: _____
Graziela Henriques Westphalen
Aluna

Ass.: _____
Profa. Dra. Luciane Macedo de Menezes
Orientadora

Ass.: _____
Profa. Dra. Maria Ivete Bolzan Rockenbach
Professora Avaliadora

Ass.: _____
Prof. Dr. Telmo Bandeira Berthold
Professor Avaliador

Ass.: _____
Prof. Dr. José Antonio Poli de Figueiredo
Coordenador do Programa de Pós-Graduação em Odontologia

ANEXO G – Carta de Aprovação do Projeto de Pesquisa pelo Comitê de Ética em Pesquisas da PUCRS

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA EM PESQUISA

OF.CEP-579/08

Porto Alegre, 09 de julho de 2008.

Senhora Pesquisadora,

O Comitê de Ética em Pesquisa da PUCRS apreciou e aprovou seu protocolo de pesquisa registro CEP 08/04231 intitulado: **"Toxicidade celular e genética dos materiais ortodônticos"**.

Salientamos que sua investigação está autorizada a partir da presente data.

Os relatórios do andamento do protocolo devem ser encaminhados a este CEP.

Atenciosamente,

p/ Prof. Dr. José Roberto Goldim
Coordenador do CEP-PUCRS

Ilma. Sra.
Dr. Luciane Macedo Menezes
Faculdade de Odontologia
N/Universidade

PUCRS
Campus Central
Av. Ipiranga, 6690 – 3ºandar – CEP: 90610-000
Sala 314 – Fone Fax: (51) 3320-3345
E-mail: cep@pucrs.br
www.pucrs.br/prppg/cep

ANEXO H – Carta de concessão da bolsa de estudos do “Programa de Doutorado no país com Estágio no Exterior” pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
Ministério da Educação - Anexos I e II - 2° Andar
Caixa postal 365
70359-970 - Brasília, DF
Brasil

CAPES

Prof. JORGE LUIS NICOLAS AUDY
PRÓ-REITOR DE PÓS-GRADUAÇÃO
PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
AV.IPIRANGA, 6681 - REITORIA - 3° ANDAR
Não Informado
PORTO ALEGRE - RS
90619900

Brasília, 29 de Agosto de 2008
Processo: BEX 3217/08-6

Prezado(a) Senhor(a),

Informamos que a Capes atendeu sua solicitação de concessão de bolsas de estudos, no âmbito do Programa de Doutorado no País com Estágio no Exterior, a (o) pós-graduando (a) GRAZIELA HENRIQUES WESTPHALEN.

Encaminhamos, em anexo, os documentos abaixo relacionados:

- carta de concessão individual;
- duas vias do Termo de Compromisso, devendo uma delas ser assinada e devolvida à Capes;
- instruções ao bolsista, que deverão ser lidas atentamente.

Atenciosamente

Maria Luiza de Santana Lombas
Coordenadora-Geral de Programas com o Exterior

ANEXO I - Carta convite para realização de parte da pesquisa no Commonwealth Scientific and Industrial Research Organisation (CSIRO) em Adelaide – Austrália

CSIRO Human Nutrition
PO Box 10041 Adelaide SC
South Australia 5000
Australia
Telephone: (08) 8303 8800 • Facsimile: (08) 8303 8899

CSIRO

Graziela Henriques Westphalen
Pontificial Catholic University of Rio Grande do Sul/ Orthodontics Departament
Av. Ipiranga, 6681 - prédio 06, s.209
Partenon - Porto Alegre
90619-900, RS - Brazil
Tel.: (+55) 51 3320 3538
Mobile phone: (+55) 51 9101 1314
E-mail: grazihw@yahoo.com.br

25 January 2008

Dear Graziela Henriques Westphalen ,

Letter of invitation

Thank you for your interest in doing part of your research on genotoxicity from metallic orthodontic appliances in Australia at CSIRO Human Nutrition Adelaide, South Australia under the supervision of Dr Michael Fenech and Dr Philip Thomas.

I am pleased to advise that you will be accepted to participate in this project subject to (i) you successfully obtaining a scholarship from the Brazilian Government covering all your travel, accommodation and laboratory costs and then (ii) obtaining an entry visa to Australia for at least the duration of the scholarship and (iii) CSIRO contract terms and conditions for this position.

As the first step I would like to invite you to submit your application to the appropriate Brazilian funding institutions and wish you good luck.

Yours sincerely,

Dr Michael Fenech
Theme Leader, Nutrigenomics and Nutrigenetics
Stream Leader, Genome Health

ANEXO J - Carta com parecer sobre pesquisa desenvolvida no Commonwealth Scientific and Industrial Research Organisation (CSIRO) em Adelaide - Austrália

Human Nutrition
Gate 13, Kintore Avenue, Adelaide SA
PO Box 10041, Adelaide BC SA 5000, Australia

Telephone: +61 8 8303 8800 • Facsimile: +61 8 8303 8899 • ABN 41 687 119 230

CSIRO

3rd. March 2009

To whom it may concern,

As a result of obtaining a Brazilian scholarship to study overseas, Graziela Henriques Westphalen spent four months full time at the CSIRO, Nutrigenomics and Genome health laboratory in Adelaide, under the supervision of Professor Michael Fenech and Dr Phil Thomas.

Graziela was part of a collaborative study investigating changes in *buccal micronucleus cytome biomarkers in relation to orthodontic appliances*. She has now been trained in all aspects of the buccal micronucleus cytome assay which is a minimally invasive assay used to determine rates of DNA damage, cell proliferation and cell death parameters in epithelial tissues.

Graziela worked very efficiently and conscientiously and met all of the objectives and goals of the study within the specified time frame. Whilst here she presented her results in two seminars and is currently preparing the study into a manuscript to submit for publication.

It has been a pleasure to supervise Graziela and we wish her all the best for her future endeavours.

Yours Sincerely,

Michael Fenech Ph.D.
Theme Leader: Nutritional Genomics - Nutrition for Healthy Genes and Minds
Principal Research Scientist: Nutritional Genomics, DNA Damage Diagnostics and Prevention
CSIRO Human Nutrition, Food Science Australia.
www.csiro.au/csiro/content/standard/ps71.html; www.csiro.au
Adjunct Professor, University of South Australia
Chair HUMN Project www.humn.org
Postal Address: CSIRO Human Nutrition PO Box 10041 Adelaide BC, SA 5000, Australia Tel +61 8 8303 8880; Mob 0488152010; Fax +61 8 83038899

8 APÊNDICES

APÊNDICE A - Termo de consentimento livre e esclarecido assinado pelos pacientes como autorização de participação na pesquisa.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Título da Pesquisa: Citotoxicidade e Genotoxicidade dos materiais ortodônticos.

I. Objetivos e justificativa da pesquisa: Essa pesquisa pretende avaliar os efeitos dos materiais que constituem os aparelhos ortodônticos no organismo humano, mais precisamente no material genético das células da boca

II. Descrição do procedimento: Células serão coletadas da sua bochecha com uma escova interdental pequena que será friccionada gentilmente.

III. Os desconfortos ou riscos esperados: O único desconforto esperado é a pressão (mínima) da escova na bochecha durante a coleta das células. As escovas utilizadas serão de uso exclusivo de cada paciente e descartadas após o uso, evitando qualquer risco de contaminação.

IV. Os benefícios que se podem obter: A obtenção de conhecimento dos efeitos dos materiais constituintes dos aparelhos ortodônticos no organismo humano.

V. Garantia de resposta a qualquer pergunta: Qualquer dúvida em relação à pesquisa estamos inteiramente disponíveis a questionamentos, nesse caso entrar em contato com: Dr^{a}. Graziela H. Westphalen ao telefone: 51 91011314 ou 3320 3538 e/ou Comitê de Ética em Pesquisa da PUCRS ao telefone: 51 33203345.

VI. Liberdade de abandonar a pesquisa sem prejuízo para si: Caso desejar abandonar a pesquisa, não haverá qualquer prejuízo ao participante ou modificações no tratamento realizado.

VII. Garantia de privacidade: Ressaltamos, também, que a concordância em participar desse estudo não implica necessariamente em qualquer modificação no tratamento que já está sendo feito. Da mesma forma, a não concordância em participar desse estudo não irá alterar de nenhuma maneira o tratamento já estabelecido.

VIII. Compromisso com informação atualizada do estudo: Os resultados da pesquisa serão transmitidos de forma atualizada aos participantes e meios científicos.

Eu, ...(paciente ou responsável) fui informado dos objetivos da pesquisa acima de maneira clara e detalhada. Recebi informação a respeito do tratamento recebido e esclareci minhas dúvidas. Sei que a qualquer momento poderei solicitar novas informações e modificar minha decisão se assim eu o desejar. A Dr^{a}. Graziela Westphalen certificou-me de que todos os dados referentes aos exames realizados serão confidenciais, bem como o respectivo tratamento não será modificado em razão desse estudo, e terei liberdade de não mais retirar consentir em participar da pesquisa, em face de essas informações.

Fui informado que não existirão danos à minha saúde causados diretamente pela pesquisa. Também sei que, caso existam gastos adicionais, estes serão absorvidos pelo orçamento da pesquisa. Caso tiverem novas perguntas sobre este estudo, posso chamar Graziela Henriques Westphalen ao telefone: 51 91011314 ou 33203538 e/ou Comitê de Ética em Pesquisa da PUCRS ao telefone: 51 33203345 para qualquer pergunta sobre os meus direitos como participante deste estudo ou se penso que fui prejudicado pela minha participação.

Declaro que recebi cópia do presente Termo de Consentimento.

_____ _____

Assinatura do Paciente Nome do Paciente

Data:

APÊNDICE B – Questionário de saúde respondido pelos pacientes participantes desse estudo.

QUESTIONÁRIO DE SAÚDE

HISTÓRIA PESSOAL

Nome:_____

Data de hoje:_____ Idade:____ Data de nascimento:_____

Sexo: ( ) Feminino ( ) Masculino

HISTÓRIA DE FUMO

Você fuma ? SIM NÃO

Se positivo, Há quanto tempo você fuma?_____

Quantas carteiras você fuma por dia? Menos de 1/2 carteira ½ - 1 carteira mais de uma carteira

HISTÓRIA DE ÁLCOOL

Você toma bebidas alcoólicas? nunca de vez em quando sempre

Quantidade:_____

HÁBITOS DIETÉTICOS

Quantas frutas você come por dia? Nenhuma, 1 a 2, 3 ou mais

Quanta salada você come nas refeições? Nenhuma, 1/6 do prato, 1/4 do prato ou mais

Quantos pedaços de carne você come por dia? Nenhum, 1 a 2 , mais de dois

Marque abaixo quais os alimentos que você consome **diariamente:**

Bolachas recheadas Salgadinhos Batatas fritas Sorvetes

HISTÓRIA GENÉTICA

Você é portador de defeito de nascimento ou de outra desordem genética ou doença inerente que afetou seus pais, irmãos ou filhos? SIM NÀO

Se SIM, favor especificar:_____

_____

DOENÇAS

Outro tipo de doença:_____

_____

HISTÓRIA DE EXPOSIÇÃO

Você foi exposto a algum dos elementos químicos (amianto, radiação, produtos do carvão, pesticidas, herbicidas, produtos do petróleo, tinturas, solventes, outros químicos)? (se positivo, responda às seguintes questões abaixo)

* Quando você foi exposto pela primeira vez? (mês e ano)_____

* Quando foi a última exposição? (mês e ano)_____

Por quanto tempo em termos de dias, meses ou anos no total você tem sido exposto?

_____

_____

_____

OBS.:_____

_____

_____

_____