Original Article

Genotoxic and cytotoxic effects of Haas appliance in exfoliated buccal mucosa cells during orthodontic treatment

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ABSTRACT

Objectives: To evaluate the genotoxic and cytotoxic effects of Haas appliances through micronuclei test and cytogenetic damage analysis in buccal mucosa epithelial cells of patients undergoing orthodontic treatment.

Materials and Methods: Twenty-eight patients, 6–12 years of age and of both genders, who required a Haas appliance for the correction of a posterior crossbite were included. Epithelial cells from the mucosa were collected by gently scraping the inside of both the right and left cheeks. The cells were collected before the insertion of the appliance (T0), 1 month after the device was installed (T1), and again 3 months after the appliance was immobilized (T2). The cells were processed to obtain slides. Feulgen/Fast Green was used as the staining method, and the number of normal, karyolytic, pyknotic, nuclear buds, bi/trinucleated, and micronucleus cells were counted under light microscopy. Cellular abnormalities were evaluated with parametric and nonparametric tests for comparison of the means by analysis of variance testing, Tukey posttest, or the Kruskal-Wallis test and then by Dunn's posttest. The significance level was 5%.

Results: There were no statistically significant changes in the micronuclei in the evaluated periods ($P > .05$). Nuclear buds increased at T1 ($P < .05$), returning to baseline levels at T2. Other abnormalities (cariolytic, pyknotic, and bi/trinucleated cells) showed a significant increase at T1 and T2 ($P < .0001$).

Conclusions: The Haas appliance did not cause an increase in micronuclei in cells of the buccal mucosa. However, statistically significant increases in cariolytic, pyknotic, and bi/trinucleated cells were observed during treatment, suggesting possible DNA damage. (*Angle Orthod.* 2018;88:590–595.)

KEY WORDS: Palatal expansion technique; Micronucleus tests; Genotoxicity; Cytogenetic

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INTRODUCTION

Posterior crossbite is a feature of malocclusion defined as a transverse reverse position of the maxillary and mandibular teeth.[1,2] Rapid maxillary expansion is an important method used to correct posterior crossbite by opening the medial palatine suture during the skeletal growth period. This is achieved through the use of heavy force with rigid and fixed devices to acquire maximum skeletal response and minimal dental movement.[1–3] Haas and Hyrax appliances are commonly used for this purpose. Components of the appliances include nickel (Ni) and chrome (Cr) screws, wires, and bands.[4] Wires and bands are attached together with silver solder. Orthodontic devices made by Ni, Cr, cobalt (Co), and beryllium (Be) have been observed to induce a genotoxic effect.[5]

The difference between Haas and Hyrax appliances is that the Haas appliance has an acrylic block that is composed of methyl methacrylate monomer, dimethyl terephthalate, copolymers of ethylene with methyl methacrylate, peroxide, and pigments. These components could add an additional genotoxic/cytotoxic damage factor for the patient.[6,7] Bonding composites and orthodontic cements used to fix the appliance in place have components such as bisphenol A–glycidyl methacrylate, urethane dimethacrylate, and triethylene glycol dimethacrylate as main monomers and comonomers.[8,9] This could also lead to mutagenic/genotoxic damage and cytotoxic effects.[8]

A potentially dangerous effect of biodegradation of orthodontic appliances is the possibility of genotoxic DNA damage.[5] Genotoxicity is the ability of an agent to exert deleterious effects on cell genetic material, affecting the cell's integrity. While cytotoxicity is the ability of a given agent to be toxic to a cell,[8] the micronucleus test (MNT) is probably the best biomarker for genetic damage[10] and is commonly used in orthodontic studies.[11–15] This assay serves as a measurement of chromosomal damage produced by clastogenic (chromosome breaks) or aneugenic agents (mitotic spindle damage).[16] Although the genotoxic effects of orthodontic appliances (mainly composites, bands, or brackets) have been studied,[8,13,16,17] the genotoxic effects of the Haas appliance have not been investigated yet. Therefore, the objective of this study was to evaluate, in vivo, the genotoxic and cytotoxic effects of the Haas appliances in exfoliated buccal mucosa cells on patients undergoing posterior crossbite treatment.

MATERIALS AND METHODS

The Research Ethics Committee (CAAE 58659616.0.0000.5419) approved this project. The patients agreed to participate in the study, and they or their guardians each signed an informed consent form.

Selection of Patients

The sample size calculation was performed based on the results observed by Gonçalves et al. (2015),[16] which evaluated the genotoxic effects of the Hyrax appliance. The calculation predicted the need for a minimum of 20 patients with a power of 0.80% and alpha of .05. This research was a longitudinal study, in which patients were recruited from the School of Dentistry of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil. A total of 28 patients were selected of both genders, 6–12 years of age, without race distinction. The inclusion criteria were patients with mixed dentition and a posterior crossbite and without any dental restorations, dental prosthetics, or previous orthodontic treatment. Not included in the study were patients who reported hypersensitivity to metals such as nickel and chrome, with syndromes or systemic diseases such as anemia, diabetes, or debilitating diseases; diseases related to genomic instability; alcoholism; smoking; drug addiction; and those who had been exposed to radiation or chemicals. Also not included were patients currently being treated with antibiotics or any type of steroids, patients using mouthwashes with alcohol within 3 months of treatment, and patients with oral diseases such as periodontitis or carious lesions.

The Haas appliances were formed with stainless steel bands (Dental Morelli, Sorocaba, SP, Brazil) placed on permanent maxillary first molars. They were constructed of silver welded onto 0.9-mm-diameter stainless steel wire (Dental Morelli, Sorocaba, SP, Brazil). Wires and bands were welded with silver solder (Dental Morelli, Sorocaba, SP, Brazil), and metallic structures were united with self-curing acrylic resin (Artigos Odontológicos Clássicos Ltda, São Paulo, Brazil). The active component was an 11-mm expansion screw (Dental Morelli).

Glass ionomer Ultra band-lok (Reliance Ortho Prod, Itasca, Ill) was used for cementation. Wires were bonded onto buccal and palatal canines or molars with light-cured composite resin Filtek^{MR} Z350 XT (3M ESPE, Campinas, SP, Brazil). After correction of the posterior crossbite, which lasted 20 days on average, the screw was stabilized with 0.0120-inch thread ligature (Dental Morelli) and fluid resin composite Natural Flow (DFL, Rio de Janeiro, RJ, Brazil). The appliance was kept in place passively for an additional 3-month retention period. During the retention period, patient evaluation was performed every 15 to 21 days

to clean the expander and remove small pieces of food localized under the acrylic.

Sample Collection

To minimize interindividual variations, individuals were observed longitudinally: each patient acted as his or her own control. Sample collections were performed at three time points: before treatment (T0), after 1 month (T1), and 3 months after stabilization (T2). Collection of cells was performed by a trained researcher and followed a standardized protocol[18] with minor modifications. First, patients were asked to rinse their mouth with a 0.9% sodium saline solution twice for 1 minute before the collection of the cells to eliminate the exfoliated dead cells. Then, epithelial cells from the buccal mucosa were collected by gentle scraping of the internal side of the right and left cheeks for 30 seconds. The cells were transferred to 15-mL plastic tubes (Falcon, LMP, Kasui, China) containing 5 mL 0.9% sodium saline solution. The tubes were kept refrigerated in ice coolers (approximately 4°C) and were then immediately transported to a laboratory for analysis.

The MNT was carried out according to Tolbert et al.[19] and Thomas et al.,[18] with minor modifications. First, the tubes containing the cells in 0.9% sodium chloride solution were centrifuged at 1000 rpm for 5 minutes. Then, part of the supernatant was removed and discarded with a pipette. The centrifugation step was repeated three times. At the end of centrifugation, the cells were suspended in a small volume of fixation solution (methanol/acetic acid 3:1; Sigma-Aldrich, St Louis, Mo). Five drops of dimethyl sulfoxide (Sigma-Aldrich) were added. This step was repeated twice. The cytologic preparations were made in duplicate and applied onto precleaned slides (Knittel Glass, Starfrost, Germany) and dried at room temperature for 24 hours. After that, the slides were stained with the Feulgen/Fast Green method (Figure 1), which is recommended as a standard buccal cell-staining protocol.[20] Finally, the slides containing the cells were analyzed under a light microscope (binocular optical microscope; Carl Zeiss, Göttingen, Germany) at 400× magnification. A single experienced investigator (Dr Cunha) performed blinded analysis and scored a total of 1000 cells. At least one slide from each period (T0, T1, and T2) was analyzed twice by the same investigator and then by a different examiner (Dr Castillo) for evaluating repeatability.

The inclusion criteria for the counting of micronuclei and the other cells anomalies followed recommendations described by Tolbert et al.,[19] with actualization realized by the HUMN and HUMNxl (www.humn.org) projects, where cytotoxic damage and other nuclear anomalies were added.[20] In addition to the values of micronuclei (MN), the presence of bi/trinucleated, pyknotic, karyolytic, and nuclear buds ("broken eggs") was evaluated.

Statistical Analysis

The data were analyzed using Prism Graphpad Prism version 7.0 for Mac (GraphPad Software Inc, La Jolla, Calif). The cellular abnormality results were expressed as mean ± standard deviation (SD) and were evaluated with parametric and nonparametric tests for comparison of the means by analysis of variance testing, followed by the Tukey posttest or by the Kruskal-Wallis test, followed by Dunn's posttest. The level of significance was 5%.

RESULTS

The characteristics of the studied sample are presented in Table 1. Among 28 patients, 17 were female (60.7%) and 11 were male (39.3%). The mean age was 9 years 3 months (SD, 18.05 months). The mean treatment period was 3 months 9 days (SD, 18.15 days). The MNT results are shown in Table 2. In normal cell analysis, there was no statistically significant difference between T0 and T1 ($P > .05$). However, a statistically significant difference was observed between T1 and T2 ($P < .05$) with values reduction. In the micronuclei analysis, a significant difference was not observed between periods ($P > .05$). In the bi/trinucleated, karyolytic, and pyknotic cell analysis, a statistically significant increase was observed among all periods ($P < .05$). A statistically significant increase was observed between T0 and T1 in nuclear bud analysis ($P < .05$). At T2, the mean values became similar to those observed at baseline; however, no statistically significant differences were observed.

DISCUSSION

Several factors may affect genotoxic results related to micronuclei, such as the type of studied appliance, appliance composition and manufacturer, sample size, treatment duration, experimental model used, and staining methods applied.[5,20,21] Different staining techniques have been used to evaluate genotoxicity by MNT. The use of nonspecific DNA staining can lead to false-positive results compared with the DNA-specific stain method. MN contain DNA; therefore, a DNA-specific staining method is essential to avoid misclassification of nonnuclear bodies, bacteria, or keratohyalin granules.[22] In the present study, the Feulgen/Fast Green technique was used. Although it is a time-consuming technique, it is a DNA-specific staining

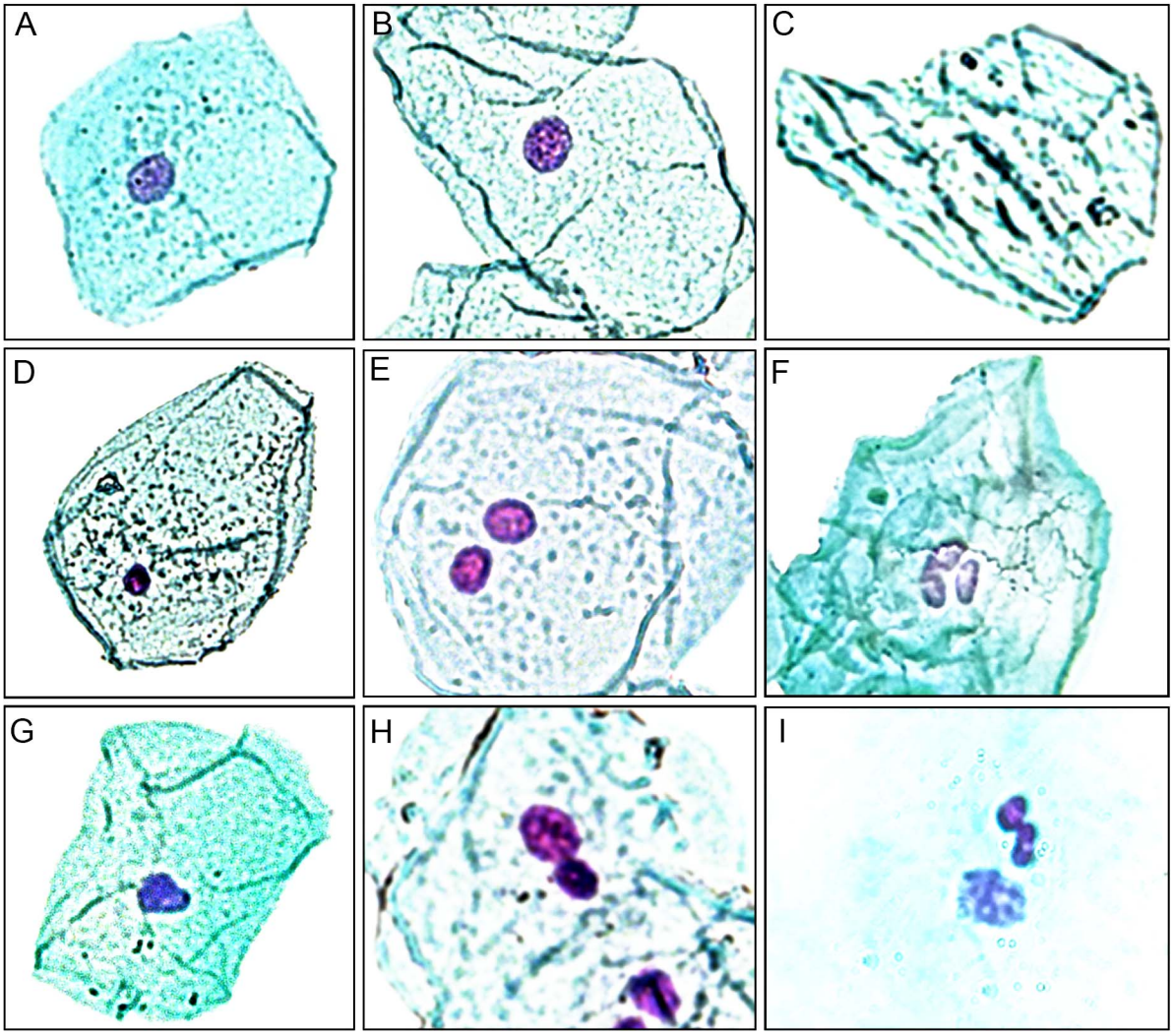

Figure 1. Cells evaluated in this study stained with the Feulgen/Fast Green method, (A, B) Normal cells. (C) Karyolytic. (D) Pyknotic. (E) Binucleated. (F) Trinucleated. (G) Nuclear buds. (H) Cell with a micronucleus. (I) Cell with two micronuclei.

method and more effective than other methods for the measurement of chromosome-damaging events in cells derived from the buccal mucosa.[16]

In orthodontic research, several studies have evaluated the genotoxicity of corrective orthodontic appliances (containing brackets, bands, wires, and composites) in exfoliated buccal mucosa cells by MNT.[8,13,15,23–25] Adding to the fact that studies used different staining methods (Papanicolaou,[23] Giemsa,[25] and Acridine Orange[8]), they also used different evaluation periods and evaluated different appliances. This may lead to conflicting results. Some authors observed an increase of MN,[13,15,23] while some authors did not observe alterations.[8,24,25] The current study did not find a statistically significant difference in MN values between periods evaluating Haas appliances, even though an increasing numerical trend was observed. Gonçalves et al.[16] found similar results using the Feulgen/Fast Green method to analyze genotoxic effects of Hyrax appliances. They showed an increasing trend of MN in the first month of treatment. Six months later, the values tended to decrease. As the trend was not statistically significant, the biological and clinical significance of these changes remains uncertain. It should be emphasized that younger patients

Table 1. Study Sample Characteristics

Patients, n	28
Gender, %	
Male	39.3
Female	60.7
Year (months)	
Mean (SD)	9.3 (18.05)
Range	82 – 143
Treatment period, d	
Mean (SD)	3.9 (18.15)
Range	98 – 183

Table 2. Comparison of Buccal Micronucleus Cytome Assay in Different Periods[a]

	Evaluation Period			
	T0	T1	T2	
Cell Type/1000 Cells	Mean (SD)	Mean (SD)	Mean (SD)	*P* Value
Normal cell	961.40 (35.05)A	959.40 (9.10)A	930.00 (13.14)B	<.0001
Pyknotic	1.17 (1.13)A	3.50 (2.18)B	6.96 (4.52)C	<.0001
Karyolytic	11.46 (3.09)A	16.39 (5.72)B	27.46 (8.05)C	<.0001
Binucleated cell	10.07 (3.72)A	15.39 (4.81)B	31.89 (7.01)C	<.0001
Nuclear bud	0.71 (1.21)A	3.50 (2.23)B	1.71 (1.21)A	<.0001
Micronuclei	0.85 (0.84)A	1.71 (1.56)A	1.75 (1.32)A	>.05

[a] Distinct uppercase letters indicate a statistical difference between periods.

showed lower levels of MN.[18] So, it is reasonable to expect that older patients using the Haas appliance may exhibit a different chromosome damage profile, justifying the absence of genotoxic changes by the micronucleus test observed in the present study, in which the age of patients was homogeneous and the sample was composed of children (6–12 years).

Regarding cytotoxicity, the values of both the pyknotic and karyolytic cells in this study increased at T1 and T2. Similar results were observed by Gonçalves et al.[16] with the Hyrax appliance. Those authors pointed out that this was possibly due to the toxic effects of the components of silver solder in contact with the buccal mucosa. The heat required during the welding procedure certainly increases the rate of corrosion.[16] Copper (Cu) present in the silver alloy leads to a higher release of toxic ions from the metals involved in the welding joint, such as Ni.[26] Binucleated cells showed increased values in the current study, with the mean slightly higher than 30/1000 cells at T2. Binucleated cells are indicative of failure in cytokinesis, associated with neurodegenerative diseases and cancer cells, suggesting that high proportions of these cells may be associated with the risk ratio for these diseases.[27] In addition, the present study showed a significant increase in nuclear bud formation at T1, but, subsequently, the mean values became similar to those observed before appliance installation. Gonçalves et al.[16] observed a similar tendency after 6 months of treatment with the Hyrax appliance. This anomaly represents a sensitive biomarker that provides direct evidence of genome damage from misrepaired DNA breaks or telomere fusion related to metal ion release.[10]

The orthodontic appliance evaluated in the present research was composed of an acrylic block resin, metallic materials welded by silver solder, wires adhered to a resinous system, and bands cemented with resin cement. This may support the hypothesis that these components, individually or in combination, may cause cell damage when the appliance is in contact with buccal mucosa. Further studies are necessary to evaluate individually the in vitro cytotoxicity of each component of the Haas appliance.

Another important factor is that the Haas appliance is in contact with the buccal cavity for shorter periods of time compared with studies examining orthodontic treatment with brackets. Although significant cytotoxic effects of the Haas appliance in exfoliated mucosa cells during their contact with the buccal cavity (about three months) were observed in the present study, studies that evaluated other orthodontic appliances over longer periods (between 6 and 9 months)[8,16,17] showed that cytotoxicity was higher in the initial periods. In addition, there is evidence of rapid residual monomers being released from orthodontic resins (24 hours) and metal ions being released in only the initial stage of the orthodontic treatment.[5] However, this rate probably decreases with time. Epithelial and epidermis cells from the buccal mucosa go through rapid turnover and regenerate generally every 7–14 days.[17] In other words, genotoxic/cytotoxic orthodontic effects may not remain in the long term. Studies have shown that device-induced changes are reversible.[5,28] However, early cytotoxic and genotoxic effects should be considered in patients genetically predisposed to additional genotoxic damage due to the individual's lifestyle.[24]

To avoid any DNA damage as described in this study, it is recommended that the appliances be constructed with the minimum amount of acrylic resin and silver solder required for adequate adaptation and retention. After activation is complete, the expander should be kept in the mouth for 3 months as retention, following the protocol proposed by Haas.[1]

CONCLUSION

- The Haas appliance did not cause an increase of micronuclei in exfoliated buccal mucosa cells, but this study demonstrated a statistically significant increase in cariolytic, pyknotic, and bi/trinucleated cells during contact of the appliance with the buccal cavity.

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