
ACTA ODONTOLOGICA LATINOAMERICANA

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Effect of bleaching protocols on surface roughness and color change of high- and low-viscosity bulk-fill composite resins

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ABSTRACT

The aim of this in vitro study was to evaluate the effect of bleaching protocols on the surface roughness (Ra), color change and surface micromorphology of a low-viscosity bulk-fill composite (Filtek Bulk Fill Flow, 3M ESPE), a high-viscosity bulk-fill composite (Filtek Bulk Fill, 3M ESPE) and a conventional nanoparticulate composite resin (control) (Filtek Z350 XT, 3M ESPE). Forty samples of each composite (disks 5 mm in diameter and 2 mm thick) were randomly divided into four groups (n=10), according to bleaching protocol: a) 10% carbamide peroxide gel (Opalescence, Ultradent Products) (2 h/day, for 14 days); b) 40% hydrogen peroxide gel (Opalescence Boost, Ultradent Products) (three bleaching sessions, once a week, 45 min/session); c) whitening rinse (Listerine Whitening Extreme, Johnson & Johnson) (2 min/day, for 14 days); and d) distilled water (control). The samples were submitted to triplicate readings (Ra and color [CIELAB parameters]) before and

after contact with bleaching protocols. Micromorphology was analyzed in a scanning electron microscope (SEM). Ra and color parameters (ΔL , Δa , Δb and ΔE) were analyzed by generalized linear models ($\alpha=0.05$). The Ra of the high-viscosity bulk-fill was significantly higher than that of the other composites ($p<0.05$). Ra increased significantly ($p<0.05$) and surface became more irregular (SEM analysis) in all the composite resins, regardless of the bleaching protocol ($p<0.05$). The high-viscosity bulk-fill composite resin group had significantly lower ΔE ($p<0.05$) than the nanoparticulate composite resin group immersed in distilled water. It was concluded that the characteristics of each resin significantly influenced the Ra more than the bleaching protocol. The high-viscosity bulk-fill resin presented minor color change.

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Keywords: bleaching agents- composite resins- surface properties.

Efeito de protocolos clareadores na rugosidade de superfície e alteração de cor de resinas compostas bulk-fill de alta e baixa viscosidade

RESUMO

Este estudo in vitro teve objetivo de avaliar o efeito de protocolos clareadores sobre a rugosidade de superfície (Ra), alteração de cor e micromorfologia de resina bulk-fill de baixa viscosidade (Filtek Bulk Fill Flow, 3M ESPE), alta viscosidade (Filtek Bulk Fill, 3M ESPE) e de uma resina composta nanoparticulada (controle) (Filtek Z350 XT, 3M ESPE). Quarenta amostras de cada resina composta (discos de 5 mm de diâmetro e 2 mm de espessura) foram aleatoriamente divididas em quatro grupos, de acordo com protocolo clareador (n=10): a) Gel de peróxido de carbamida (Opalescence, Ultradent Products) (2 horas/dia, por 14 dias); b) Gel de peróxido de hidrogênio (Opalescence Boost, Ultradent Products) (3 sessões de clareamento, uma por semana, 45 min/sessão); c) enxaguatório clareador (Listerine Whitening Extreme, Johnson & Johnson) (2 min/dia, por 14 dias); d) água destilada (controle). As amostras foram submetidas a leituras, em triplicata (Ra e cor [parâmetros CIELab]) antes e depois do contato

com os protocolos clareadores. A micromorfologia de superfície foi conduzida em microscópio eletrônico de varredura (MEV). Ra e parâmetros de cor (ΔL , Δa , Δb e ΔE) foram analisados por modelos lineares generalizados ($\alpha=0.05$). A Ra da resina bulk-fill de alta viscosidade foi significativamente superior do que para os outros compósitos ($p<0.05$). A Ra aumentou significativamente ($p<0.05$) e a superfície ficou mais irregular (MEV) para todos os compósitos, independente do protocolo clareador ($p<0.05$). A resina bulk-fill de alta viscosidade obteve menor ΔE ($p<0.05$) do que a resina composta nanoparticulada, imersa em água destilada. Pode-se concluir que as características de cada resina composta influenciaram de forma mais significativa a Ra do que o protocolo clareador. A resina bulk fill de alta viscosidade apresentou menor alteração de cor.

Palavras-chave: agentes clareadores- resinas compostas- propriedades de superfície.

INTRODUCTION

Due to their mechanical and aesthetic properties, composite resins have been widely used for direct

restorations on anterior and posterior teeth¹. However, they still have some adverse consequences as a result of polymerization contraction, which

is aggravated by cavities with a high cavity configuration factor such as those occurring in posterior tooth cavities². Polymerization contraction may lead to cusp deflection, adhesive interface cracks, postoperative sensitivity and staining at the restoration margins^{3,4}. One way to minimize the stresses caused by polymerization contraction is by inserting conventional composite resins into cavity preparations using the incremental technique, in which small increments of composite resin are placed in the cavity, with minimal contact between opposing walls⁵. However, this technique takes longer and poses the risk of empty spaces forming between increments⁶.

In this respect, bulk-fill resins were developed with the alternative of inserting a single increment of up to 4 mm or 5 mm, and the advantages of providing reduced polymerization shrinkage and requiring less time to perform posterior tooth restorations⁶⁻⁹. Polymerization shrinkage was reduced by incorporating components capable of interacting with the photoinitiator and modulating polymerization kinetics, providing an additional photoinitiator, and promoting relatively slower conversion, longer pre-gel phase, and gel delay^{10,11}. Bulk-fill resins come either in fluid consistency (flowable or low viscosity), which must be coated with a conventional composite resin except in the proximal region, or paste consistency (high-viscosity), which does not require occlusal restoration coverage¹², but which may be subject to degradation due to direct interaction with the oral environment.

On the other hand, in response to patients' demand for lighter-colored teeth, dental professionals recommend tooth bleaching. This is an effective conservative technique¹³ that can be performed in different ways. One option is the home bleaching technique in which the patient uses trays at home, supervised by the dentist. Home bleaching uses 5.5% to 10% hydrogen peroxide concentrations or 10 to 22% carbamide peroxide concentrations. Another option is the in-office technique, in which the bleaching product is applied by the professional, using 35% to 40% hydrogen peroxide concentrations^{14,15}. During the bleaching procedure, the bleaching agents may come into contact with pre-existing oral cavity restorations, and may affect their surface roughness^{16,17} and surface micromorphology¹⁸. Although this problem is more serious in anterior restorations, bleaching gel is often applied in posterior regions, such as premolars, and

may reach the occlusal and proximal face of these teeth and therefore, posterior restorations. Thus, considering the possibility that the monomers in bulk-fill resins may be eluted after contact with bleaching gels¹⁹, it is essential to investigate the effects of bleaching agents on the mechanical properties of conventional and bulk-fill resins, especially those of high viscosity.

In addition to dentist-supervised bleaching techniques, some over-the-counter (OTC) bleaching products can be sold to the consumer without a dentist's supervision. These products contain hydrogen peroxide concentrations of about 3%²⁰, and include mouthwashes, toothpaste, chewing gum and whitening strips²¹. There is a lack of clinical evidence regarding the safety and efficacy of these products, even though they are used very frequently and without proper supervision, making them potentially harmful. Whitening rinses, in particular, may lead to an increase in the surface roughness of conventional composite resins²². However, the literature includes few studies evaluating the effects of over-the-counter bleaching products, such as mouthwashes containing hydrogen peroxide, on surface roughness and micromorphology and on color change in high- and low-viscosity conventional and bulk-fill resins.

Thus, the aim of this *in vitro* study was to evaluate the effect of applying bleaching protocols (office, home or OTC) at different concentrations on surface roughness and micromorphology, and on color change of high- and low-viscosity bulk-fill resins, compared to conventional nanoparticulate resin. The null hypothesis (H_0) tested was that the different bleaching protocols applied would not affect the surface roughness and micromorphology or change the color of the tested composite resins.

MATERIALS AND METHODS

Ethical Aspects

The present study was exempted from ethical approvals because it did not involve animals or human subjects (Exemption protocol: 2018/0931).

Preparation of samples

Forty disk-shaped specimens were prepared in a silicone mold (5 mm in diameter and 2 mm thick) from each of three types of composite resins (see Table 1 for further details):

Table 1. Composition of materials used in the experiment

Material Lot	Composition* (% by weight)	pH**
Conventional composite resin (Filtek Z350 XT, 3M ESPE, St. Paul, MN, USA) 772615	Diurethane dimethacrylate (UDMA) (1 - 10) Bisphenol A polyethylene glycol diether dimethacrylate (Bis-EMA) (1 - 10) Bisphenol A diglycidyl ether dimethacrylate (Bis-GMA) (1 - 10) Polyethylene glycol dimethacrylate (PEGDMA) (< 5) Triethylene glycol dimethacrylate (TEGDMA) (< 1) Silane treated ceramic (60 - 80) Silane treated silica (1 - 10) Silane treated zirconia (1 - 5)	--
Low-viscosity bulk-fill composite (Filtek Bulk Fill Flow, 3M ESPE, St. Paul, MN, USA) N4862A3	Silane treated ceramic (50-60) Diurethane dimethacrylate (UDMA) (10-20) *substituted dimethacrylate (10-20) Bisphenol A polyethylene glycol diether dimethacrylate (Bis-EMA) (1 - 10) Ytterbium Fluoride (1-10) Bisphenol A diglycidyl ether dimethacrylate (Bis-GMA) (1 - 10) Benzotriazol (< 1) Triethylene glycol dimethacrylate (TEGDMA) (< 1) Ethyl 4-Dimethyl Aminobenzoate (EDMAB) (<1)	--
High-viscosity bulk-fill composite (Filtek Bulk Fill, 3M ESPE, St. Paul, MN, USA) N693115	Silane treated ceramic (60-70) Aromatic urethane dimethacrylate (10 - 20) Ytterbium fluoride (1 - 10) Silane treated silica (1 - 10) 1,12-dodecane dimethacrylate (DDDMA) Silane treated zirconia (< 5) Water (< 5) Pentanedioic acid, 2,2-dimethyl-4-methylene, reaction products with glycidyl methacrylate (< 1) Ethyl 4-dimethyl aminobenzoate (EDMAB) (< 0.5) Benzotriazol (< 0.5) Titanium dioxide (< 0.2)	--
10% Carbamide peroxide gel (Opalescence PF, Ultradent Products, Inc., South Jordan, USA) D04J0	Carbamide peroxide (<20) Poly acrylic acid (<10) Sodium hydroxide (<5) Sodium fluoride (0.25)	6.8
40% Hydrogen peroxide gel (Opalescence Boost PF Ultradent Products, Inc., South Jordan, USA) D04GG	Hydrogen peroxide (<40) Glycerin (<12) Potassium nitrate (<3) Potassium hydroxide (<30) Sodium Fluoride (<10)	7.3
Whitening rinse Listerine Whitening Extreme (Johnson & Johnson, USA) 2387COM	Water Alcohol Hydrogen peroxide (2.5) Poloxamer 407 Sodium Saccharin Menthol Phosphoric acid Disodium phosphate Sodium fluoride (100 ppm) Sucralose	3.48

* Composition of composite resins and bleaching products according to the MSDS (Material Safety Data Sheet) provided by the manufacturer, except for the whitening rinse, whose composition was extracted from the packaging.
 ** pH values of bleaching products were measured in triplicate with a pH meter.

- Conventional composite resin (Filtek Z350 XT, 3M ESPE™ Dental Products, St. Paul, MN, USA)
 - Low-viscosity bulk-fill flowable composite resin (Filtek Bulk Fill Flow, 3M ESPE™ Dental Products, St. Paul, MN, USA)
 - High-viscosity bulk-fill composite resin (paste) (Filtek Bulk Fill, 3M ESPE™ Dental Products, St. Paul, MN, USA).
- The composite resin was inserted into the silicone mold with a #1 silicate spatula (Golgran, São Caetano do Sul, SP, Brazil). The resin surface was

covered with a polyester strip and a glass slide to ensure a flat surface and to achieve standard compression of the composite resin. A 500g weight was applied over the whole set for 60 s.

After the weight was removed, photopolymerization was performed with a LED photoactivator (Valo, Ultradent Products Inc, S. Jordan, UT, USA) for 20s (standard mode, 1000 mW / cm² power), by placing the tip of the device on the glass slide. Finishing and polishing were performed with the complete orange sequence of Soflex disks (3M ESPE™ Dental Products, St. Paul, MN, USA), according to decreasing grain. Each disk was used for 10 s and then changed.

Bleaching Protocols

The specimens made from each composite resin were divided randomly into four subgroups (n = 10), to which different surface treatments were applied, as follows:

- Home bleaching technique with 10% carbamide peroxide gel (Opalescence, Ultradent Products Inc, S. Jordan, UT, USA) – the bleaching agent was applied to the sample surface for 2 hours a day, and this protocol was repeated for 14 days²³.
- Office technique with 40% hydrogen peroxide gel (Opalescence Boost, Ultradent Products Inc, S. Jordan, UT, USA). Three bleaching sessions were performed at one-week intervals. In each session, the bleaching agent was applied to the sample surface for 15 min and this procedure was repeated 3 times. The timing and frequency of application were as recommended by the manufacturer.
- Immersion in a hydrogen peroxide-containing whitening rinse (Listerine Whitening Extreme, Johnson & Johnson, New Jersey, USA). In this group, the resin samples were immersed in 2 mL of bleaching solution for 2 min, once a day, and then placed on a seesaw agitator (Model: K40-3012, Kasvi Import and Distribution of Laboratory Products, Curitiba, Paraná, Brazil). This protocol was repeated for 14 days. The application time for each cycle was as recommended by the manufacturer.
- Control group - immersion in distilled water for 14 days, with daily water renewal.

Between the bleaching protocol cycles, all samples were immersed in distilled water and stored in an oven at 37°C (Bacteriological oven - Odontobrás Ind. e Com. Med. Odont.; Model: ECB 1.3 Ribeirão Preto, SP, Brazil).

Surface roughness, color change and surface micromorphology were evaluated before and after the bleaching protocols.

Surface roughness test

A roughness meter (SurfTest SJ-210, Mitutoyo Corporation, Kanagawa, Japan) was used to measure surface roughness. Three random measurements were made on the surface of each sample, with a 0.25 mm cut off, at a speed of 0.1 mm/s¹⁶. An average value was assigned to each sample for each timepoint.

Color evaluation

Color was measured with a spectrophotometer (Vita Easyshade, Vita-Zahnfabrik, Germany), using the CIELAB color system. The detector was positioned perpendicular to the surface of the specimens to measure the parameters of luminosity (L*), red-green coordinate (a*), and yellow-blue coordinate (b*). The analysis was performed in a box, so that there would be no influence of the external environment, over a white background.

After completion of the experimental periods, the *L, *a, *b parameters were measured again, and the values of ΔL, Δa and Δb color change were calculated. The total color change expressed in ΔE is represented by the following formula:

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2} \quad 24$$

Surface micromorphology evaluation

Three samples from each group were prepared for surface micromorphology analysis. The samples were gold-plated (Sputter Coater EMITECH, K450, Kent, UK) and examined under scanning electron microscopy (Scanning Electron Microscope, SEM: Leo 440i, EDS: 6070, LEO Electron Microscopy / Oxford, Cambridge, England) to obtain the images. Each sample showed the full extent of the surface run at 500x magnification, performed by a single, previously calibrated operator. A representative area was selected to characterize what occurred in most of the sample surface and photographed at 1000x magnification.

Statistical Analysis

Surface roughness data and color variables (ΔL, Δa, Δb and ΔE) did not meet the assumptions of a parametric analysis. Thus, the surface roughness data were analyzed by generalized linear models for repeated measures over time, with the factorial resin

vs. bleaching protocol. The delta values (Δ) were analyzed by generalized linear models, considering the resins vs. bleaching protocols as the factorial scheme. The analyses were performed on R programs (Core Team, R Foundation for Statistical Computing, Vienna, Austria) and SAS programs (SAS Institute Inc. SAS® Studio 3.5: User's Guide, Cary, NC: SAS Institute Inc) with a significance level of 5%.

RESULTS

Surface roughness was found to be higher in high-viscosity bulk-fill resin, and lower in low-viscosity bulk-fill resin, regardless of the bleaching protocol (Table 2). All the bleaching protocols promoted increased surface roughness, regardless of the composite resin.

Color analysis showed that the low-viscosity bulk-fill resin had a statistically lower ΔL and a negative value when 10% carbamide peroxide gel was applied, compared with the other bleaching protocols ($p < 0.05$) (Table 3). As for the high-viscosity bulk-fill resins and conventional composite resins, there was no significant difference among the bleaching protocols regarding ΔL ($p > 0.05$).

The group of low-viscosity bulk-fill resins submitted to the 10% carbamide peroxide gel protocol showed a statistically lower Δa ($p < 0.05$) than the group receiving the 40% hydrogen peroxide gel application, or the group immersed in whitening rinse, but was statistically similar to the group immersed in distilled water (Table 3). For the high-viscosity bulk-fill

composite resin, there was no significant difference among the bleaching protocols for Δa ($p > 0.05$) and for the conventional resin; statistically higher Δa was observed in the group immersed in whitening rinse than in the other bleaching protocols ($p < 0.05$).

For the low-viscosity bulk-fill resin, a statistically higher Δb value was observed for the group immersed in distilled water, in which 10% carbamide peroxide gel was applied, than for the other bleaching protocols (Table 3). For the high-viscosity bulk-fill resin, Δb did not differ significantly among the bleaching protocols ($p > 0.05$). For the conventional resin, Δb was significantly higher when the resin was immersed in whitening rinse than with the other bleaching protocols ($p < 0.05$).

The low-viscosity bulk-fill resin showed significantly greater variation in total color (ΔE) when 10% carbamide peroxide gel was applied. The high-viscosity bulk-fill resin presented higher ΔE in water than when subjected to the 40% hydrogen peroxide gel protocol ($p < 0.05$). The conventional composite resin showed significantly higher ΔE after bleaching with 10% carbamide peroxide gel, and when in contact with the whitening rinse ($p < 0.05$) (Table 3).

For illustrative purposes, the surface morphology of the sample was analyzed by scanning electron microscope, and the influence of bleaching protocols on the resins tested was observed. All composite resins tested showed altered surface morphology, with deepening of furrows and grooves, regardless of the bleaching protocol used (Fig. 1).

Table 2. Median (minimum and maximum values) of surface roughness (Ra) according to composite resin, bleaching protocol and time.

Timepoint	Bleaching Protocol	Composite resin		
		Low-viscosity bulk-fill composite	High-viscosity bulk-fill composite	Conventional composite resin (control)
Baseline	Distilled water (control)	0.24 (0.15-0.30) Ca	0.45 (0.24-0.74) Aa	0.35 (0.27-0.49) Ba
	10% Carbamide peroxide gel	0.22 (0.03-0.37) Ca	0.40 (0.07-0.88) Aa	0.26 (0.06-0.58) Ba
	40% Hydrogen peroxide gel	0.26 (0.05-0.31) Ca	0.45 (0.29-0.80) Aa	0.30 (0.08-0.76) Ba
	Whitening rinse	0.25 (0.11-0.36) Ca	0.36 (0.18-0.46) Aa	0.28 (0.18-0.46) Ba
After-bleaching protocol	Distilled water (control)	*0.30 (0.15-0.43) Ca	*0.58 (0.38-0.87) Aa	*0.37 (0.19-0.57) Ba
	10% Carbamide peroxide gel	*0.26 (0.20-0.40) Ca	*0.48 (0.24-0.77) Aa	*0.42 (0.25-0.73) Ba
	40% Hydrogen peroxide gel	*0.28 (0.19-0.44) Ca	*0.54 (0.29-0.99) Aa	*0.34 (0.16-0.56) Ba
	Whitening rinse	*0.30 (0.26-0.43) Ca	*0.42 (0.32-0.88) Aa	*0.40 (0.21-0.64) Ba

* Value differs from the time after the sample preparation (baseline) ($p \leq 0.05$). Medians followed by distinct letters (uppercase horizontally and lowercase vertically within each timepoint) differ from each other ($p \leq 0.05$).

$p(\text{resin}) < 0.0001$; $p(\text{bleaching protocol}) = 0.6297$; $p(\text{resin vs. bleaching protocol}) = 0.8884$; $p(\text{time}) < 0.0001$; $p(\text{resin vs. time}) = 0.0059$; $p(\text{bleaching protocol vs. time}) = 0.3762$; $p(\text{resin vs. bleaching protocol vs. time}) = 0.8116$.

Table 3. Median (minimum; maximum) of the parameters of evaluation of the color change (ΔL , Δa , Δb and ΔE) according to composite resin, bleaching protocol and time.

Variable	Bleaching Protocol	Composite resin		
		Low-viscosity bulk-fill composite	High-viscosity bulk-fill composite	Conventional composite resin (control)
ΔL	Distilled water (control)	1.47 (-0.80; 3.43) Aa	0.78 (-1.73; 3.13) Aa	1.00 (0.07; 2.87) Aa
	10% Carbamide peroxide gel	-2.23 (-4.63; 2.87) Cb	-0.27 (-1.77; 2.47) Ba	2.15 (-1.40; 3.93) Aa
	40% Hydrogen peroxide gel	1.85 (-4.70; 5.73) ABa	0.70 (-3.63; 1.83) Ba	2.08 (0.13; 2.97) Aa
	Whitening Rinse	0.33 (-4.33; 7.07) Ba	1.43 (-3.03; 2.87) ABa	2.18 (-3.27; 3.90) Aa
Δa	Distilled water (control)	1.30 (0.90; 1.67) Aab	1.32 (0.23; 3.10) Aa	0.98 (0.13; 2.70) Ab
	10% Carbamide peroxide gel	0.97 (0.13; 2.17) Bb	0.72 (0.37; 1.43) Ba	1.77 (-0.77; 3.50) Ab
	40% Hydrogen peroxide gel	1.77 (-0.10; 2.60) Aa	1.18 (0.50; 1.83) Aa	1.47 (0.73; 2.53) Ab
	Whitening rinse	1.63 (-0.17; 4.30) Ba	0.62 (0.23; 2.40) Ca	2.63 (0.63; 5.77) Aa
Δb	Distilled water (control)	-4.97 (-8.17; -4.20) Cb	-0.50 (-10.37; 1.60) Ba	1.28 (-2.23; 5.67) Ab
	10% Carbamide peroxide gel	-7.00 (-8.50; -4.80) Cb	-2.30 (-3.80; -0.43) Ba	3.17 (-3.87; 7.90) Ab
	40% Hydrogen peroxide gel	-2.90 (-6.90; -0.03) Ca	-0.87 (-3.83; 0.63) Ba	2.00 (-0.73; 6.10) Bb
	Whitening Rinse	-3.23 (-6.97; -0.87) Ca	-0.73 (-3.77; 2.00) Ba	4.73 (1.03; 8.97) Aa
ΔE	Distilled water (control)	5.80 (4.81; 8.29) Ab	2.92 (1.35; 10.74) Ba	2.60 (1.38; 6.15) Bb
	10% Carbamide peroxide gel	7.77 (5.44; 8.98) Aa	2.54 (1.22; 3.86) Cab	4.48 (2.15; 9.49) Ba
	40% Hydrogen peroxide gel	4.59 (3.10; 8.51) Ab	1.68 (1.27; 5.31) Cb	3.17 (1.37; 6.57) Bb
	Whitening Rinse	4.98 (3.64; 7.26) Ab	2.21 (1.32; 4.35) Bab	6.23 (4.08; 9.93) Aa

Medians followed by distinct letters (uppercase horizontal and lowercase vertically) differ from each other ($p \leq 0.05$).

ΔL : $p(\text{resin})=0.0004$; $p(\text{bleaching protocol})=0.0336$; $p(\text{resin vs. bleaching protocol})=0.0035$.

Δa : $p(\text{resin})=0.0003$; $p(\text{bleaching protocol})=0.0398$; $p(\text{resin vs. bleaching protocol})=0.0122$.

Δb : $p(\text{resin})<0.0001$; $p(\text{bleaching protocol})<0.0001$; $p(\text{resin vs. bleaching protocol})=0.0189$.

ΔE : $p(\text{resin})<0.0001$; $p(\text{bleaching protocol})=0.0021$; $p(\text{resin vs. bleaching protocol})<0.0001$.

DISCUSSION

According to the results of the present study, the null hypothesis was rejected, since there were significant differences in roughness, surface micromorphology and color among the composite resins after being subjected to the bleaching protocols.

Surface roughness (R_a) was statistically lowest for the low-viscosity bulk-fill resin and highest for the high-viscosity bulk-filled resin, with an intermediate value for conventional nanoparticulate resin, whether at the initial timepoint (baseline) or after application of the bleaching protocols. This may be attributed to the polishing system used for the samples, as reported by Rigo *et al.*²⁵, who compared the same bulk-fill resins as those in the present study. They reported that the smoothness of the R_a of the resins was promoted by the polyester tape, and was

statistically similar among the resins. However, when using the Soflex system (3M ESPE), they showed that R_a was higher for the high-viscosity bulk-fill resin than for the low-viscosity bulk-fill resin. This can be explained by the composition of the high-viscosity bulk-fill resin, in which the new monomers that were introduced (such as AUDMA and DDDMA) may have been more eluted, resulting in greater abrasion of the organic matrix during polishing, and consequent exposure of the surface loads and increased R_a ²⁵. It can be assumed that alternative polishing systems may promote different results.

R_a increased significantly in all resins after bleaching with any of the protocols. SEM showed that irregularities and grooves in the micromorphology increased after the bleaching protocols, possibly due

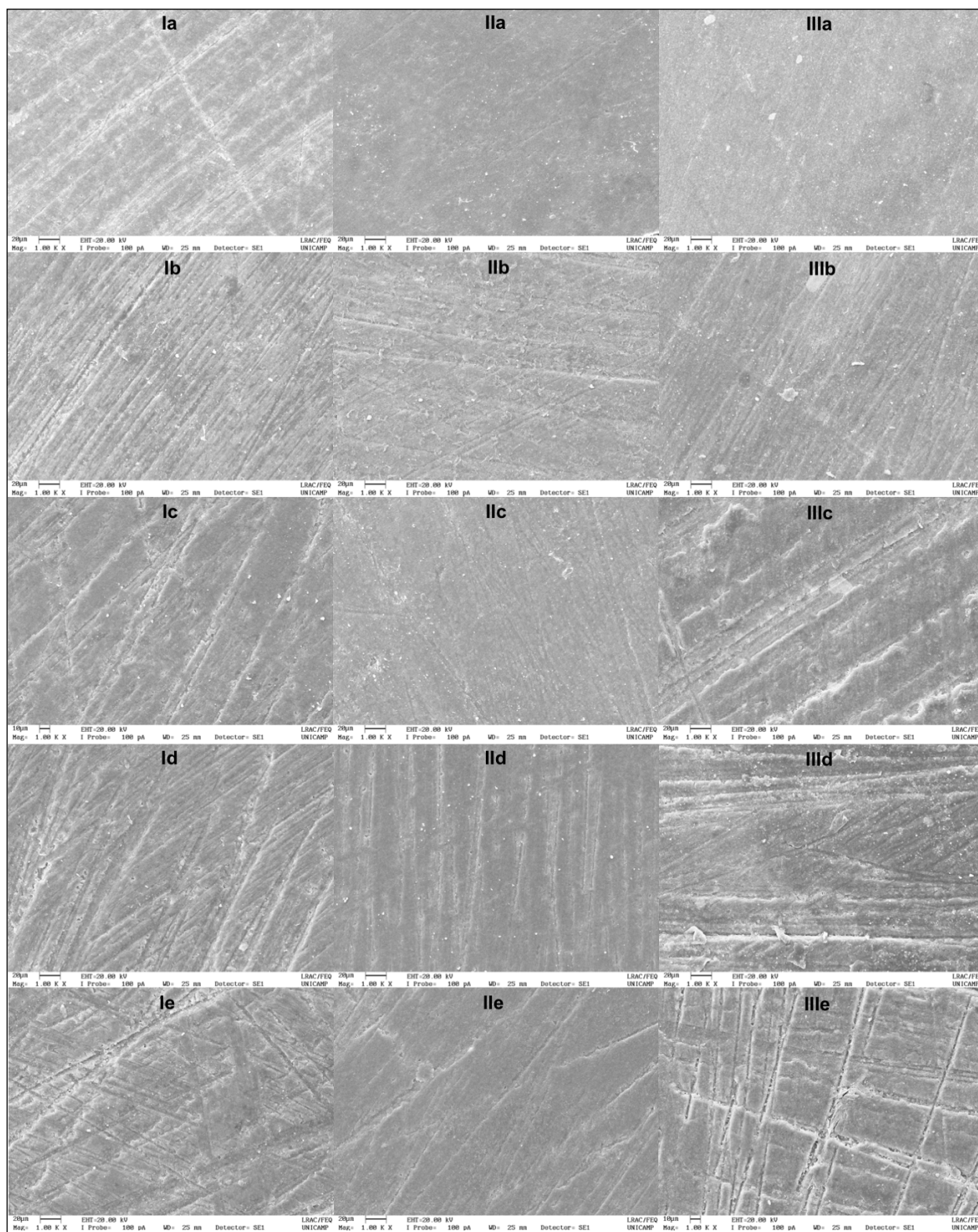


Fig. 1. Surface Micromorphology (SEM analysis). I. Conventional composite resin; II. Low-viscosity bulk-fill composite; III. High-viscosity bulk-fill composite; a. baseline; b. Whitening rinse; c. 40% hydrogen peroxide gel; d. 10% Carbamide peroxide gel and e. Distilled water.

to structural changes in the composite resins caused by the action of peroxides^{18,26-28} and water sorption. Water sorption may soften the matrix and degrade the composite resin surface, with consequent exposure of the inorganic load and increased Ra²⁹⁻³¹. Color analysis found that the low-viscosity bulk-fill resin had statistically lower ΔL and Δb values than the other resins when submitted to the 10% carbamide peroxide bleaching agent and to the other bleaching protocols. This may be associated with the effect of 10% carbamide peroxide on the surface, since it is associated with higher translucency of low-viscosity bulk-fill resin. Thus, constant daily application may have led to chromatic change of the resin, represented clinically by a darkening and blueness of the material. It is worth noting that low-viscosity bulk-fill resin is not exposed to the buccal environment except on the proximal face, since the manufacturer recommends that it be coated with conventional composite resin. Thus, from a clinical standpoint, color change in this type of resin may be less relevant than in high-viscosity bulk-fill resins and conventional resins.

ΔE was high in the conventional nanoparticulate composite resin, especially after immersion in whitening rinse and 10% carbamide peroxide. This may be associated with the significantly higher Δb and the positive value of this nanoparticulate composite resin under these conditions, demonstrating that there was yellowing of conventional composite resin samples. These changes may be related to the structural composition of the organic matrix of the conventional resin, which has a higher hydrophobic monomer content, but which has hydrophilic characteristics, in the case of BisGMA and TEGDMA. Both monomers have water sorption capacity when in contact with aqueous solutions³², which may have some effect on color change. These results were more significant when the composite resin was subjected to the home bleaching protocol with 10% carbamide peroxide, and especially with the whitening rinse protocol. Thus, it is believed that the daily contact of these agents with the resin surface may have increased the degradation of the organic matrix and exposure or detachment of the inorganic load³³, which may have altered and contributed to greater color change. It should also be emphasized that whitening rinses contain phosphoric acid and alcohol in their composition, as well as having acidic pH. These substances may have had deleterious

effects on the surface of the conventional composite resin, promoting the color change.

The color change was also high for low-viscosity bulk-fill resins. This may be explained by the similarity of the composition of these resins to the conventional composite resin, which includes BisGMA and TEGDMA monomers. It was observed that the high-viscosity bulk-fill composite resin had less color change than the conventional composite resin, as reported by Mansouri and Zidan³⁴. This result may be related to the composition of the high-viscosity bulk-fill resin, because of the presence of UDMA and AUDMA monomers, as well as the absence of Bis-GMA and TEGDMA. UDMA is a monomer that has higher hydrophobia³⁵ and lower sorption and water solubility³⁰ than the Bis-GMA and TEGDMA monomers present in Z350 and low-viscosity bulk fillers.

Several authors consider 3.3 a critical value for ΔE , considering it is clinically unacceptable, because the color modification at this rate becomes perceptible to the human eye^{31,36-38}. In this study, the low-viscosity bulk-fill resin presented values greater than 3.3, regardless of the protocol tested, whereas the high-viscosity bulk-fill resin presented values lower than 3.3, regardless of the protocol tested. It was concluded that the high-viscosity bulk-fill resin material provides greater color stability.

Within the limits of this study, it was observed that the tested agents influenced the color of the restorative materials. Dentists should be aware that pre-existing restorations may change significantly after tooth bleaching, and replacement may be necessary.

Moreover, it is believed that phenomena such as greater Ra and alterations in color and in surface micromorphology occur more markedly in the oral cavity, because of the ionic composition of saliva, the ingestion of acidic foods and beverages, and the presence of acids from bacterial metabolism³⁹, and should be considered. Furthermore, brushing exerts an effect on optical properties of bulk fill composites,⁴⁰ and this was not simulated in the present study. Thus, further research and clinical trials are needed to prove the effects of different bleaching protocols applied to high- and low-viscosity bulk-fill resins.

It was concluded that the characteristics of each composite resin influenced the surface roughness more significantly than the bleaching protocol. After the resins came into contact with the

bleaching protocols and water, roughness increased and surface morphology changed. In general, the greatest color change in the low-viscosity bulk-fill resin was observed after contact with 10% carbamide peroxide gel, whereas the greatest color

change in the Z350 nanoparticulate composite resin was promoted by the bleaching protocol with 10% carbamide peroxide gel and hydrogen peroxide whitening rinse. Thus, higher color stability was observed for high-viscosity bulk-fill resin.

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Expansion treatment using Invisalign®: Periodontal health status and maxillary buccal bone changes. A clinical and tomographic evaluation.

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ABSTRACT

The aim of this study was to evaluate changes in periodontal status and maxillary buccal bone by considering clinical and tomographic parameters during the first year of orthodontic expansion with Invisalign® aligners. Upper first (1PM) and upper second (2PM) premolars of 19 patients with orthodontic expansion requirement treated with Invisalign® aligners were evaluated. Plaque index (PI), gingival index (GI), probing pocket depth (PPD), clinical attachment level (CAL) and cone beam tomographic (CBCT) records were collected at 76 sites before starting treatment (T0) and at 12 months (T1). Bone height was measured from cemento-enamel junction (CEJ) to the crest cortical bone (CC). Bone thickness was measured at two levels: 4 mm (CEJ+4) and 6 mm (CEJ+6) apical to the CEJ. A descriptive analysis was made of the variations of bone thickness and height in a series of cases. The average expansion was 1.93 mm for 1PM and 1.67 mm for 2PM. Arithmetic mean

of distance CEJ-CC in 1PM was 3.05 mm at T0, and remained at 3.05 mm at T1. Arithmetic mean of distance CEJ-CC in 2PM was 2.06 mm at T0 and 2.31 at T1. Post-expansion, most of the analyzed sites (86%) exhibited a bone thickness of ≥ 0.5 mm. The greatest variations between T0 and T1 were observed at the level of 1PM CEJ+4 and 2PM CEJ+6. The minimal changes in the clinical records (GI, PI, PPD and CAL) between T0 and T1 were compatible with the maintenance of gingival-periodontal health. Invisalign® for expansion movements did not produce substantial changes in the evaluated periodontal clinical parameters or in the bone measurements. Removable appliances reduce plaque retentive factors and favor adequate oral hygiene.

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Keywords: clear aligner appliances- periodontium- cone beam computed tomography- facial bones.

Tratamiento de expansión con Invisalign®: estado de salud periodontal y cambios óseos maxilares. Evaluación clínica y tomográfica.

RESUMEN

El objetivo de este estudio fue evaluar los cambios en el estado periodontal y hueso facial maxilar a través de parámetros clínicos y tomográficos durante la expansión ortodóncica con alineadores Invisalign® en el primer año de tratamiento. Se evaluaron los primeros (1PM) y segundos (2PM) premolares superiores pertenecientes a 19 pacientes con requerimiento de expansión ortodóncica tratados con alineadores Invisalign®. Se registraron los índices de placa (IP), índice gingival (IG), profundidad al sondaje (PS) y nivel de inserción (NI) y registros tomográficos de haz cónico (CBCT) en 76 sitios antes de comenzar el tratamiento (T0) y a los 12 meses (T1). Se midió la altura ósea desde el límite amelocementario (LAC) hasta la cortical de la cresta (CC) y el espesor en dos niveles; a 4 mm (LAC+4) y a 6 mm (LAC+6) hacia apical del LAC. Se realizó un análisis descriptivo de las variaciones de la altura y espesor óseo en una serie de casos. La expansión promedio para 1PM fue de 1,93 mm y para 2PM fue de 1,67 mm. La media

aritmética de LAC-CC en primeros premolares fue de 3,05 mm en T0 y se mantuvo el valor de 3,05 mm en T1. La media aritmética de LAC-CC en segundos premolares fue de 2,06 mm en T0 y 2,31 en T1. Post expansión, la mayoría de los sitios (86%) analizados exhibieron un espesor óseo $\geq 0,5$ mm. Las mayores variaciones entre T0 y T1 se observaron a nivel de 1PM CEJ+4 y 2PM CEJ+6. Los registros clínicos (PI, GI, PPD y CAL) evidenciaron mínimos cambios entre T0 y T1, compatibles con el mantenimiento de la salud gingivo-periodontal. El uso de Invisalign® para movimientos de expansión no produjo cambios sustanciales en los parámetros clínicos periodontales evaluados ni en las mediciones óseas. La aparatología removible reduce los factores retentivos de placa bacteriana y facilita una adecuada higiene oral.

Palabras clave: alineadores ortodóncicos transparentes- periodoncio- tomografía computarizada de haz cónico- huesos faciales.

INTRODUCTION

The number of adult patients requiring orthodontic treatment has increased noticeably in recent decades and Clear Aligner Therapy (CAT) has become an option for patients requiring comfort and esthetics consistent with current lifestyle¹⁻³.

The relationship between orthodontic treatments and periodontal health has always been a matter of concern to orthodontists and periodontists, whether as a result of the forces applied or due to the changes produced by orthodontic movement in both the bone and the soft tissues^{4,5}. Mild or moderate dental crowding, narrow arches or crossbites can be resolved by dentoalveolar expansion. The literature has shown that dentoalveolar expansion is the movement that most compromises the buccal bone⁶⁻⁹. Technological advances in imaging have enabled detailed evaluation of facial bone by using cone beam computed tomography (CBCT)^{3, 10-12}.

The effects on alveolar bone and soft tissues in patients treated with fixed orthodontic appliances were extensively evaluated with two-dimensional radiographs until the advent of computed tomography⁴. Mandelaris mentioned that the American Academy of Periodontology Consensus of 2017 agreed that CBCT is the only radiographic mode that enables objective detection and prediction of changes in the facial bone and the general structure of the alveolar bone¹⁰. While there is limited evidence supporting routine use of CBCT in orthodontic-periodontal therapy, experts agree that its use should be considered in various scenarios, including adult patients with dentoalveolar deficiencies (crowding and/or transverse discrepancies) which require expansion, thin periodontal phenotype and recessions, among others⁸.

Considering that there is plentiful evidence of the presence of bone dehiscence and fenestrations in patients who have never undergone orthodontic treatment, it is very important to diagnose these conditions in order to minimize risk during expansion^{13, 14}. Some studies have shown that maxillary facial alveolar bone decreases after dentoalveolar expansion when fixed appliances are used¹⁵.

There are several studies on the thickness of the buccal plate of maxillary incisors and canines, but relatively few on the thickness of the facial plate of premolars. Results vary according to the method used for measuring. The facial plate is significantly

thinner for the upper first premolar than for the upper second premolar, due to root morphology and more anterior position in the arch, among other factors^{5, 11, 12, 16-22}. Average alveolar bone thickness in the premolar zone is 1.13 mm measured at 1 mm apical to the crest, and 1.03 mm at mid-root²³.

Although CBCT can provide plentiful information, it is also essential to record gingival-periodontal clinical parameters periodically. The treatment plan should evaluate the risk of alterations at gingival level as well as attachment loss. Periodontal health should be considered as an important factor in the success of an orthodontic treatment.

Oral hygiene procedures have major impact on periodontal health during orthodontic treatment. The literature supports the link between increase in bacterial plaque indexes and decline in oral health conditions in patients under orthodontic treatment, especially when fixed appliances are used. CAT can minimize the negative orthodontics-related effects on health by enabling simpler oral hygiene procedures^{2, 24-27}.

The aim of this study was to evaluate changes in periodontal status and alveolar bone in a series of cases by considering clinical and tomographic parameters during the first year of orthodontic expansion with Invisalign® aligners.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the Argentine Society of Orthodontics on August 1, 2016. All patients signed the informed consent form accepting to participate voluntarily in the clinical study. The study protocol was in agreement with the Helsinki declaration of 1975, reviewed in 2013.

Twenty-three patients with orthodontic expansion indication and high esthetic demand, who were referred by the Admissions Department of the Argentine Society of Orthodontics from 2016 to 2018, were evaluated for treatment with Invisalign® aligners. Nineteen of these patients met the inclusion criteria and were selected to participate in the study: 16 females and 3 males, 15 to 50 years of age.

Inclusion criteria were: complete dentition, mild or moderate tooth crowding, with indication of expansion, with healthy or normalized periodontium, non-smokers. Exclusion criteria were: crowns in the upper jaw, extensive restorations, presence of carious lesions, pregnancy, presence of systemic diseases contraindicating orthodontic treatment,

history of head and neck radiation, regular use of steroids or any other medication affecting bone turnover.

All patients received an oral hygiene protocol: Bass Technique with soft toothbrush (Colgate® Slimsoft) use of dental floss, interdental brushes or picks. Use of electric toothbrush was not allowed. Recommended frequency for tooth cleaning was twice a day.

After recording periodontal indexes and performing tomographic studies, orthodontic treatment with aligners was begun. Patients were scheduled once a month for follow-up. Aligners were to be used 22 hours a day and changed every 15 days.

For this study, in the 19 selected patients, the upper first (1PM) and upper second (2PM) premolars were analyzed. Clinical and tomographic measurements were taken before beginning expansion treatment (T0) and at 12 months (T1).

Clinical records

The following indexes were recorded for buccal and proximal surfaces of upper first and second premolars:

- Löe and Silness plaque index (PI)²⁸
- Silness and Löe gingival index (GI)²⁹
- Probing pocket depth (PPD)
- Clinical attachment level (CAL)

Measurements were taken with a periodontal probe marked in millimeters (North Carolina; Hu Friedy®, Chicago IL) and rounded to the nearest millimeter on the probe scale.

Analysis of tomographic images

Facial bone thickness and height in maxillary premolars were examined by using CBCT, which provides 0.123 mm image resolution, enabling precise linear measurements with low exposure to radiation. Thicknesses under 0.12 mm might not be detected. Tomographic images were taken with 3D Accuitomo (J. Morita MFG Co., Kyoto, Japan). The scans were taken in a single 360-degree rotation at a scan time of 17 seconds, 90 Kvp, 0.125 mm voxel size and 60 x 60 mm field of view (FOV). Patient head was positioned locating Camper's Plane parallel to the ground and the midsagittal plane perpendicular to it. Position was held by headrest, headband and chinrest.

After the CBCT scans, a protocol was established to capture pre- and post-treatment images of selected teeth (1.4, 1.5, 2.4 and 2.5) using One Volume

software (J Morita MFG Co. Kyoto, Japan) in order to evaluate any changes in height and thickness of the facial bone.

Standardization of positioning of the tooth to be evaluated

A standardized orientation was established, and a protocol was followed.

1. Axial-guided navigation was used as reference to locate landmarks.
2. On the axial section, the image was positioned such that the vertical was tangential to the vestibular surface of the selected teeth, while the major axis coincided with the horizontal
3. On the coronal plane, the image was positioned such that the line passing through the buccal and palatal cemento-enamel junctions coincided with the horizontal axis

Both sections were adjusted simultaneously (Fig 1).

Measurements

Image files were processed in a conventional computer using Image J (NIH) software on a Lenovo monitor with 1280 x 1024 pixel resolution. All measurements were taken by a single examiner (VM).

The following measurements were taken on the coronal section (Fig 2):

- Distance from the cemento-enamel junction (CEJ) to the alveolar crest (CC): CEJ-CC
- Thickness of facial bone 4 mm apical to the CEJ: CEJ+4
- Thickness of facial bone 6 mm apical to the CEJ: CEJ+6

Transverse measurements

Digital models from Align Technology Clincheck® were obtained at T0 and T1. Linear values of upper arch width were measured for interfirst and intersecond premolars.

Figs 3 and 4 show clinical, virtual models and tomographic images at T0 and T1.

RESULTS

The average expansion was 1.93 mm (range 0.5 to 5.1 mm) for 1PM and 1.67 mm (range 0.3 to 3.9 mm) for 2PM. Seventy-six teeth (n=76), corresponding to upper 1PM and 2 PM were evaluated in the 19 patients.

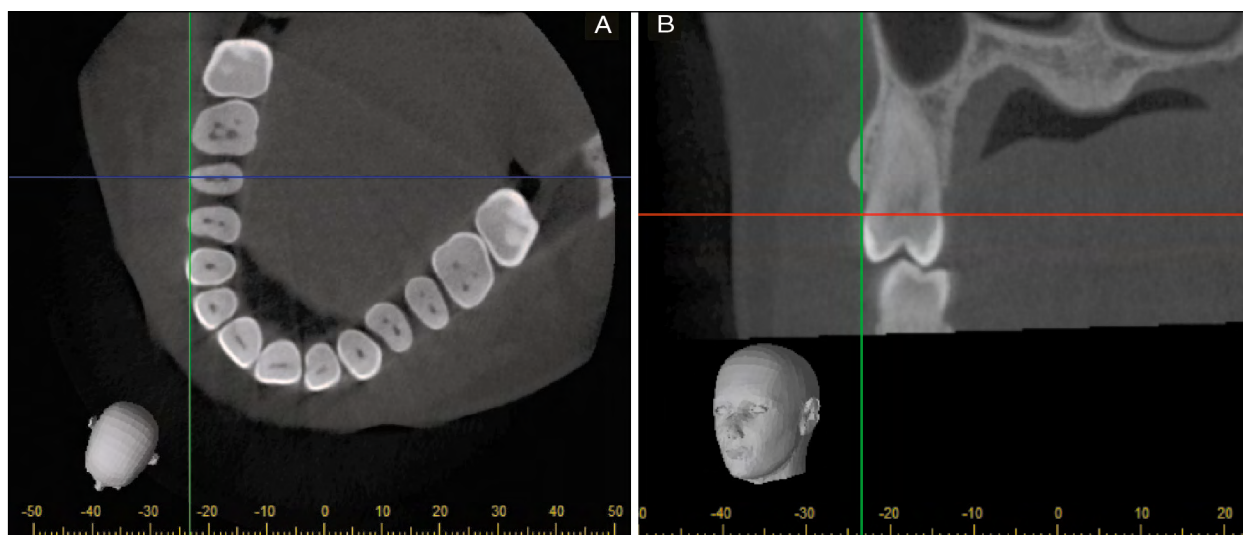


Fig. 1: a) Axial section: teeth main axis parallel to horizontal reference. b) Coronal section: line joining vestibular and palatine cements coincides with horizontal axis.

Arithmetic mean distance CEJ-CC in 1PM was 3.05 mm (range 1.09 mm to 4.99 mm) at T0, and remained at 3.05 mm at T1 (range 0.41 mm to 5.57 mm). Arithmetic mean distance CEJ-CC in 2PM was 2.06 mm (range 1.04 mm to 4.66 mm) at T0 and 2.31 (range 1.07 to 5.23) at T1.

For a descriptive analysis of the distance CEJ-CC, results were grouped into two categories (Table 1):

- Positive response (difference between T0 and T1 ≤ 0.5 mm)
- Negative response (difference between T0 and T1 > 0.5 mm).

Of the 38 1PM, 31 (81.6%) showed a difference ≤ 0.5 mm between T0 and T1. This variation indicates that the distance CEJ-CC remained the same or decreased, a positive response. Seven 1PM (18.4%) showed a difference > 0.5 mm between T0 and T1, a negative response. Of the 38 2PM, 26 (68.4%) showed a difference ≤ 0.5 mm between T0 and T1 and 12 2PM (31.6%) showed a difference > 0.5 mm between T0 and T1.

Initial facial bone thickness for 1PM ranged from 0.00 mm to 2.04 mm (average 0.80 mm) at CEJ+4 and from 0.40 mm to 2.85 mm (average 0.88 mm) at CEJ+6. For 2PM, it ranged from 0.00 mm to 3.06 mm (average 1.59 mm) at CEJ+4 and 0.60 mm to 3.94 mm (average 1.61 mm) at CEJ+6.

For analysis of bone thickness, the following categories were established (Table 2):

- Undetectable bone wall
- Thickness < 0.5 mm
- Thickness ≥ 0.5 mm

Analysis of the post-expansion bone thickness showed the following: Of the 38 1PM, at CEJ+ 4, of 31 sites with thickness ≥ 0.5 mm (T0), 28 remained in that group and in 3 sites no detectable wall was found. One site with thickness < 0.5 mm (T0) increased its thickness to ≥ 0.5 mm. Of 6 sites with no detectable wall (T0), 1 increased its thickness. At CEJ+ 6, of 34 sites with thickness ≥ 0.5 mm (T0), 32 remained in that group and at 2 sites the thickness decreased to < 0.5 mm. Of the 4 sites with thickness < 0.5 mm (T0), 2 increased their thickness to ≥ 0.5 mm. In 2PM, at CEJ+ 4, of 36 sites with thickness ≥ 0.5 mm (T0), only one site had an undetectable wall. Of 2 sites with undetectable thickness (T0), one increased its thickness to ≥ 0.5 mm. In CEJ+ 6, of the 38 sites with thickness ≥ 0.5 mm (T0), 32

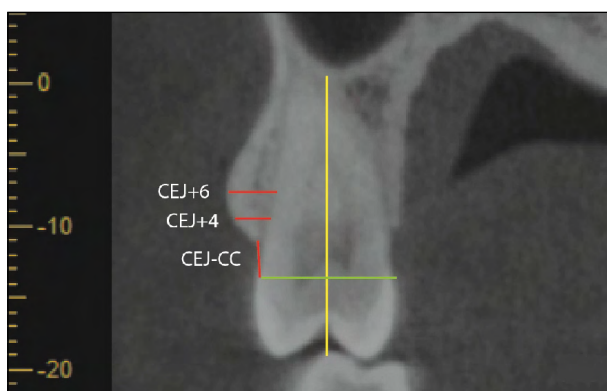


Fig. 2: Coronal section of upper premolars: distance from cemento-enamel junction to alveolar crest (CEJ-CC) and thickness of facial bone 4 and 6 mm apical to the CEJ (CEJ+4 and CEJ+6, respectively).

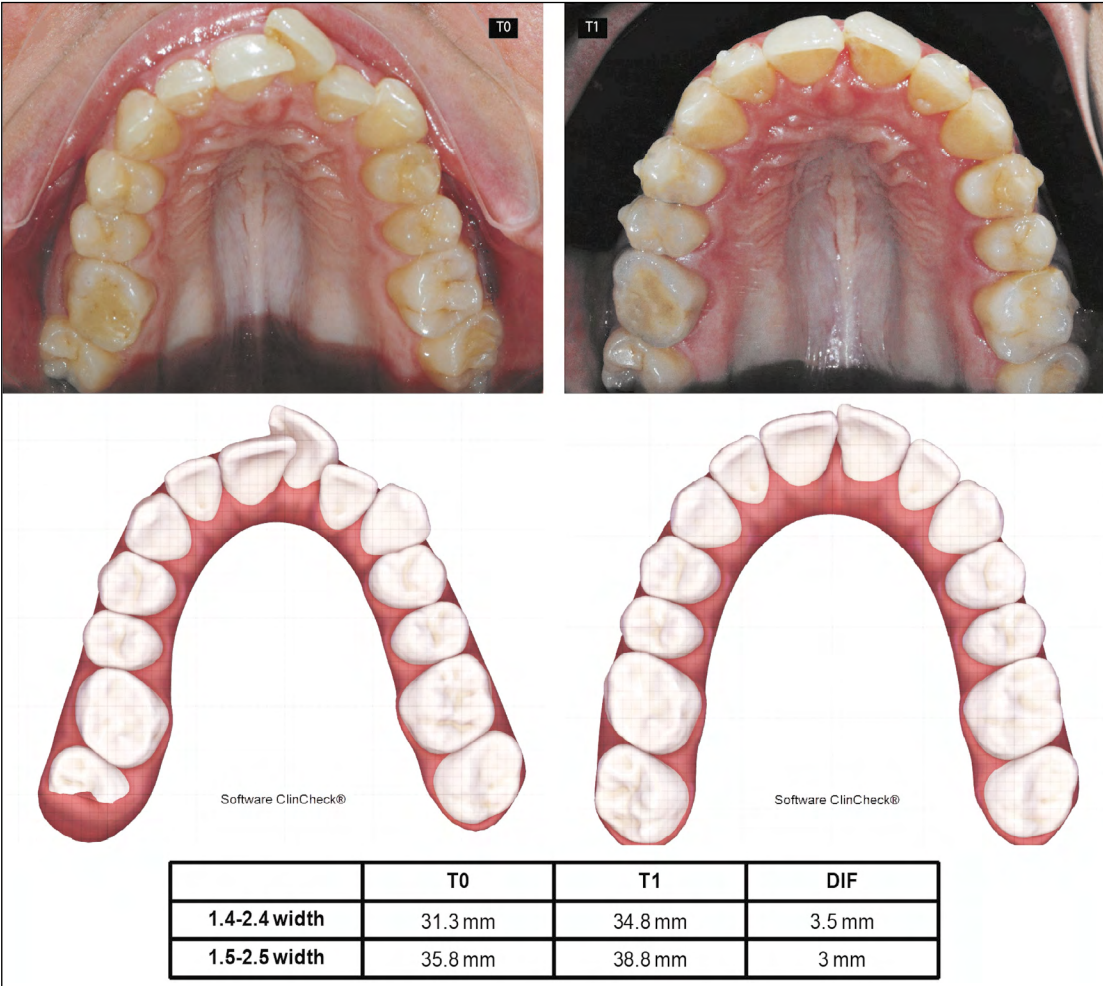


Fig 3: Clinical and virtual models (Clincheck®) images showing transverse changes between T0 and T1.

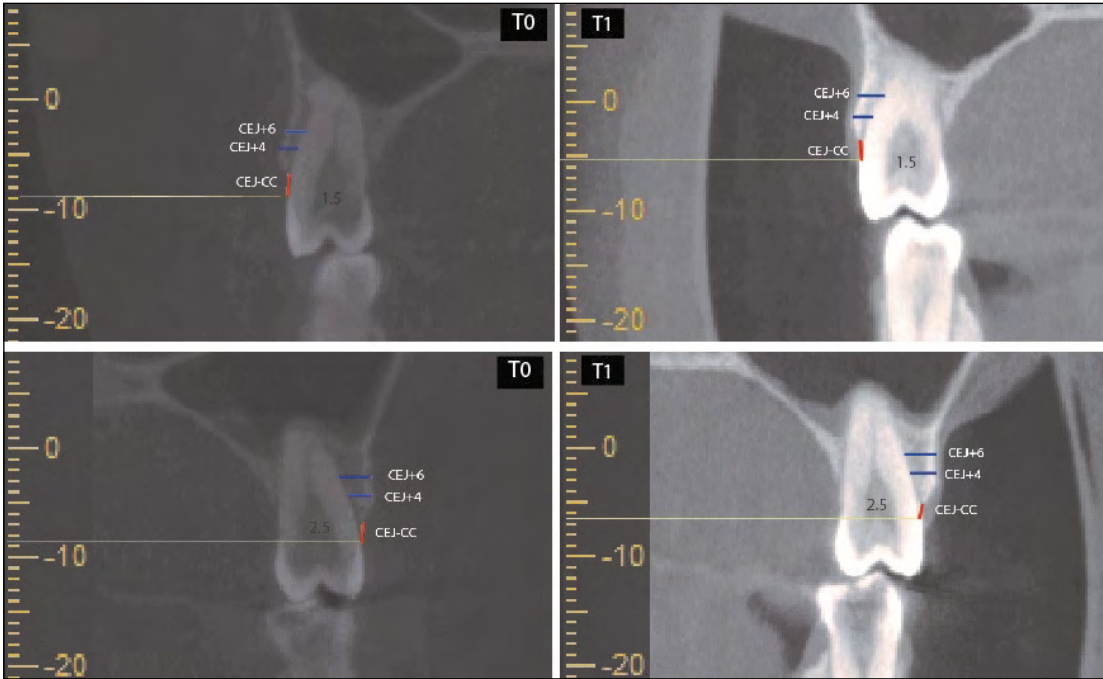


Fig 4: Tomographic images at T0 and T1.

Table 1. Changes in CEJ-CC measurements

	Positive response ≤0.5 mm		Negative response >0.5 mm	
	Sites	%	Sites	%
1PM	31	81.6	7	18.4
2PM	26	68.4	12	31.6

remained in that group, 5 became <0.5 mm thick and one was not detectable.

Figs. 5 and 6 provide data for bone height and thickness at the beginning of treatment and post-expansion.

The minimal changes in the clinical records (GI, PI, PPD and CAL) between T0 and T1 were compatible with the maintenance of gingival-periodontal health (Figs. 7 and 8). Regarding CAL, of the 76 evaluated sites, 6 lost insertion (4 of them lost 1 mm and the other 2 lost 2 mm).

DISCUSSION

The aim of this study was to analyze the changes in periodontal health status and the effects on maxillary facial bone after the first year of orthodontic expansion using the Invisalign® clear aligner therapy. Although we found some studies that analyzed alveolar bone height and/or thickness, none of them correlated this information with clinical parameters^{7,15}.

The presence of facial bone – both height and thickness – is crucial because it supports the gingival margin, providing it with stability over time, and determines its esthetic outline. If this wall is lost, bone dehiscence or gingival recessions may develop. It is therefore essential to evaluate its dimensions prior to beginning orthodontic therapy with expansion (either bodily or tipping movement).

Information provided by CBCT is useful for making the diagnosis and designing an appropriate treatment plan with an expansion requirement. The thickness of the bone plate that covers teeth in buccal and palatal aspects is one of the limiting factors of tooth movement⁴.

In our study, facial bone thickness and height in maxillary premolars were examined by using CBCT, which provides 0.123 mm image resolution, enabling precise linear measurements with low exposure to radiation. Thicknesses under 0.12 mm might not be detected.

Ali et al.⁹ reported that the range of dental expansion should be 2-3 mm per quadrant to reduce the risk of gingival recession. In the present study, the prescribed amount of expansion for each participant was customized based on measurements of the dentition and CBCT, in order to be within a safe range and to design an accurate treatment plan. The average expansion was 1.93 mm for 1PM (range 0.5 to 5.1 mm) and 1.67 mm for 2PM (range 0.3 to 3.9 mm).

In our study, facial bone height was measured by evaluating the distance from the CEJ to the CC, and its thickness was measured at two levels: CEJ+4 and CEJ+6, in 38 first upper premolars and 38 upper second premolars. For 1PM, our average value for the CEJ-CC distance was 3.05 mm at T0, similar to the value found in a study by Vera²³ (2.79 mm), who analyzed facial bone wall dimensions in anterior maxillary arch between and including 1PM. Wang et al.⁵ also analyzed facial bone wall dimensions of maxillary anterior teeth and premolars using CBCT, and their findings were lower than Vera's and ours. Height was measured from CEJ to CC and ranged from 0.1 mm to 4 mm; the mean distance was 2.2 ±0.7 mm for 1PM and 2.0 ±0.6 mm for 2PM.

Table 2. Descriptive analysis of the sample according to bone thickness at T0 and T1.

	1PM CEJ+4		1PM CEJ+6		2PM CEJ+4		2PM CEJ+6	
	T0	T1	T0	T1	T0	T1	T0	T1
	Sites	Sites	Sites	Sites	Sites	Sites	Sites	Sites
UNDETECT. WALL	6 (15.8%)	8 (21%)	0 (0%)	0 (0%)	2 (5.3%)	1 2.6	0 (0%)	0 (0%)
THICKNESS <0.5 mm	1 (2.6%)	1 (2.6%)	4 (10.5%)	4 (10.5%)	0 (0%)	0 (0%)	0 (0%)	5 (13.1%)
THICKNESS ≥0.5 mm	31 (81.6%)	29 (76.4%)	34 (89.5%)	34 89.5	36 (94.7%)	37 (97.4%)	38 (100%)	33 (86.9%)

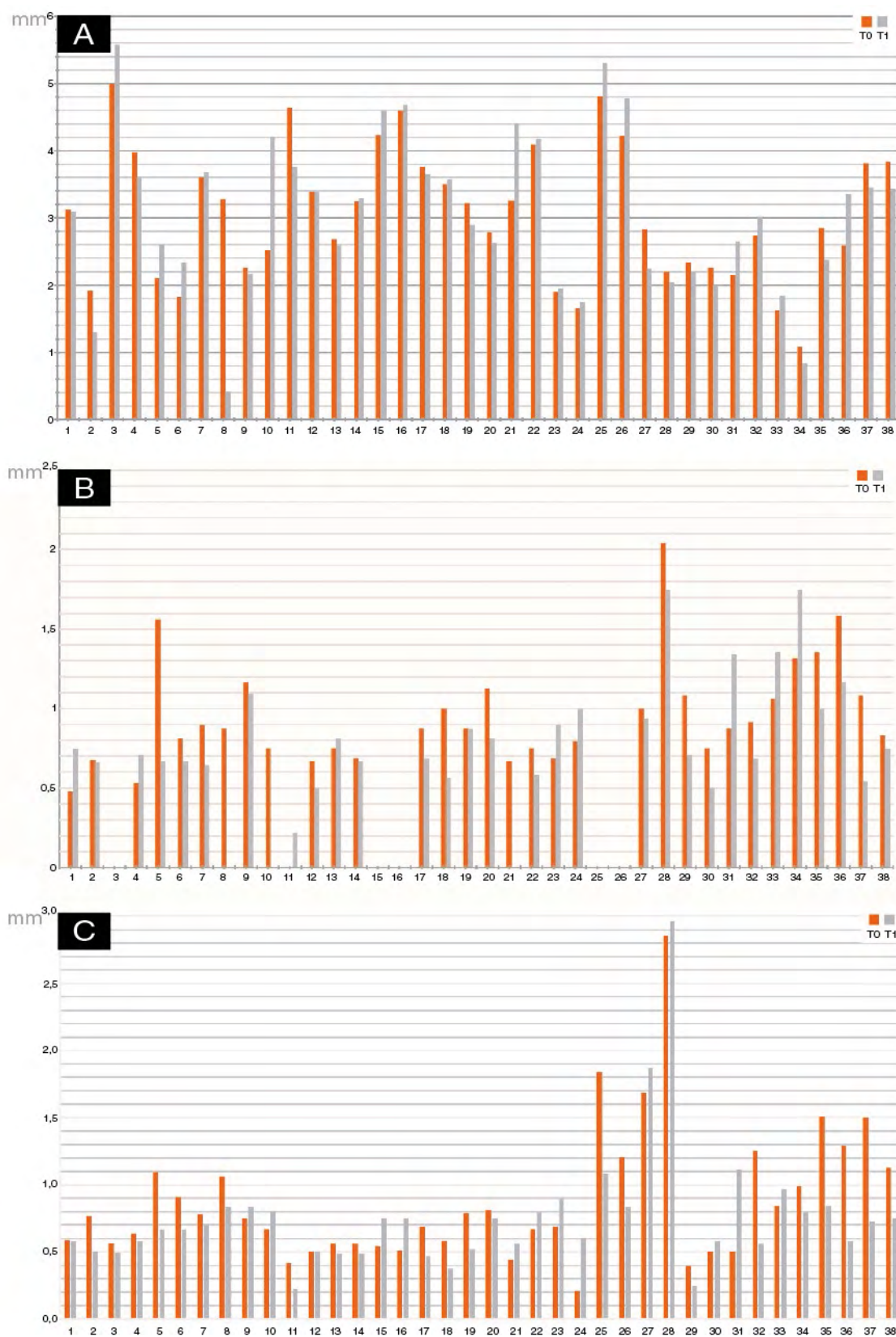


Fig 5: Tomographic measurements in 1PM at T0 and T1 for all teeth studied. A) Distance CEJ-CC. B) Facial plate thickness at CEJ+4. C) Facial plate thickness at CEJ+6



Fig 6: Tomographic measurements in 2PM at T0 and T1 for all teeth studied. A) Distance CEJ-CC. B) Facial plate thickness at CEJ+4. C) Facial plate thickness at CEJ+6

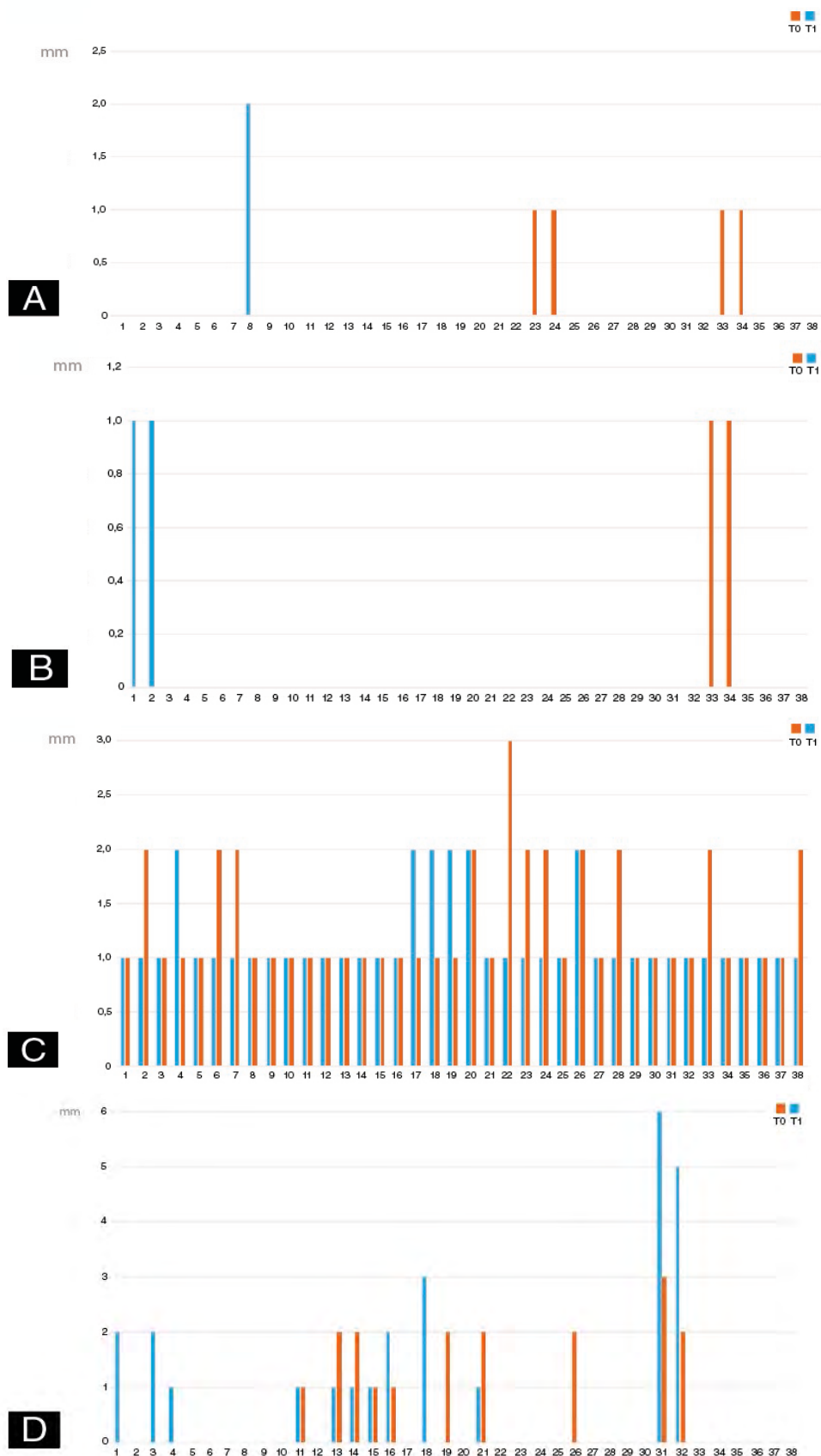


Fig 7: Clinical records in 1PM at T0 and T1 for all teeth studied. A) Gingival index (GI). B) Plaque index (PI). C) Probing pocket depth (PPD). D) Clinical attachment level (CAL)

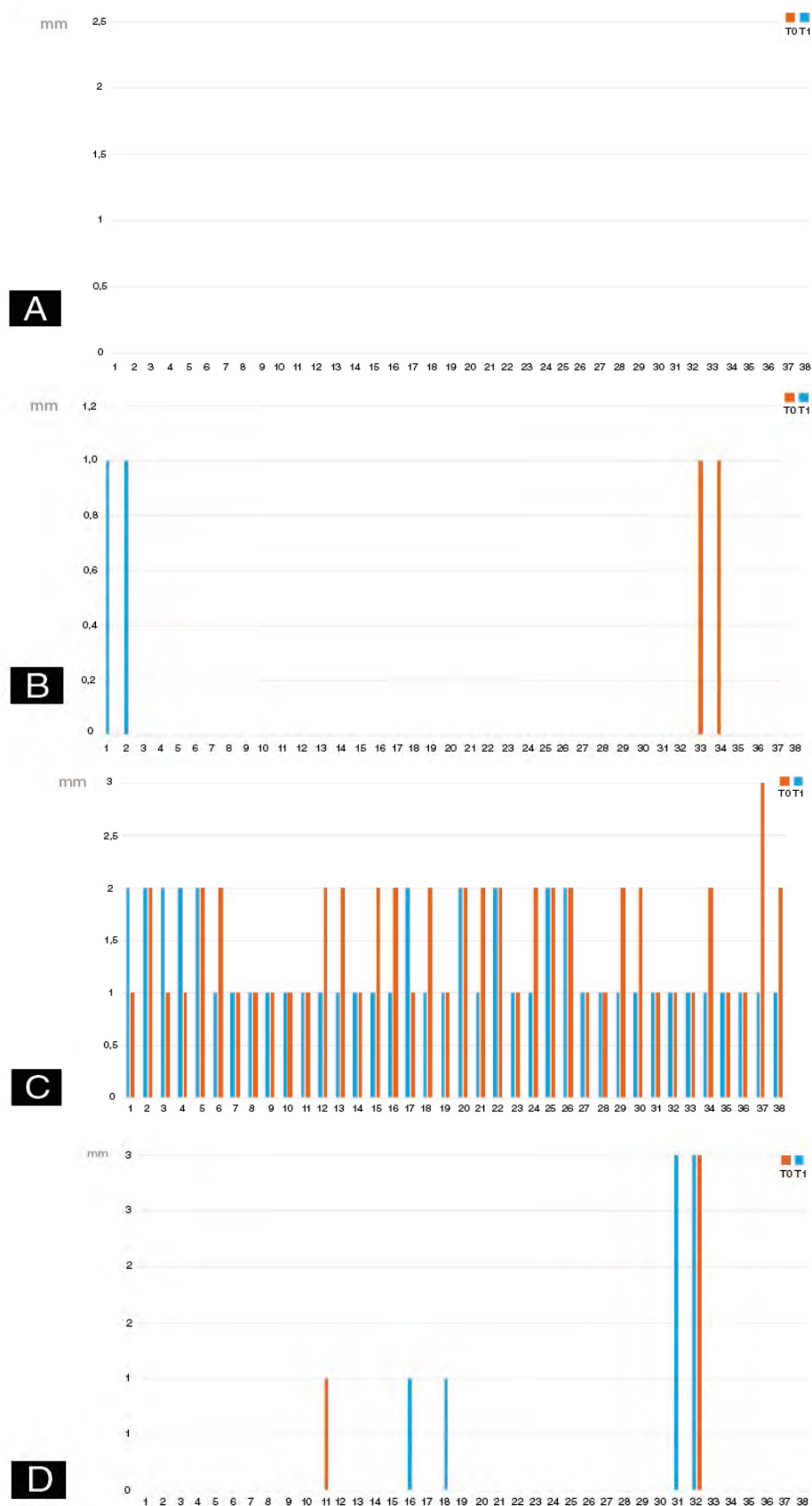


Fig 8: Clinical records in 2PM at T0 and T1 for all teeth studied. A) Gingival index (GI). B) Plaque index (PI). C) Probing pocket depth (PPD). D) Clinical attachment level (CAL)

Post-expansion for 1PM, the CEJ-CC distance remained unchanged or diminished at 31 of the 38 evaluated sites, meaning that the level of insertion remained the same or increased at these sites, considered to be a positive response. However, it increased at 7 sites, reflecting a decrease in the level of insertion. For 2PM, there were 26 sites with a positive response, while 12 showed a decrease in level of insertion. According to Castro et al.³, the association between orthodontic treatment and changes in the distance between CEJ-CC has been widely studied, but the difference in orthodontic techniques, the various criteria for radiographic evaluation, and the several methods of diagnostic imaging used in the different studies have limited the comparison of results¹².

For facial bone thickness, Vera²³ reports that the median buccal alveolar bone thickness 1 mm apical to the alveolar bone crest was 1.13 mm; and at the midroot it was 1.03 mm in the premolar area. Temple et al.¹⁸ analyzed the thickness at 1mm, 3mm and 5mm apical to CC of the posterior teeth, finding an average of 0.72 mm for 1PM at 3mm and 1.24mm for 2 PM. In our study, the initial values for 1PM (average 0.80 CEJ+4 and 0.88 CEJ+6) and for 2PM (average 1.59 CEJ+4 and 1.61 CEJ+6) agree with Temple et al.¹⁸, being lower than 1 mm, with many of them having a wall thinner than 0.5mm.

Braut et al.¹¹ analyzed the thickness of buccal bone of anterior teeth including 1 PM at two locations: 4mm and midroot. They found absence of buccal wall in 17.5% of the cases for 1PM measured at 4mm to the CEJ. Average thickness was 0.73mm at 4mm and 0.75mm at midroot. Similarly, in our sample, no buccal wall was detected in 15.8% of 1PM, and the thickness measurements were similar. Cattaneo et al.¹⁵ assessed transverse changes in upper arch as a result of using passive and active self-ligating brackets. They performed greater transversal increase in the interfirst premolar width (4.3 mm and 4.5 mm) and intersecond premolar width (4 mm and 3.3 mm) respectively; thus, they found the buccal bone area for 2PM decreased on average 14 to 20% post-dentoalveolar expansion. The buccal bone for 1PM was so thin that the error of the method surpassed the mean measured value, for which reason only the bone in the 2PM region was evaluated.

In our study, for 2PM, there was a slight increase in thickness at CEJ+4 (2.7% improvement), while

there was a reduction of 13.1% at CEJ+6 at T1. The different measurement methods, statistical evaluation, appliances and expansion amounts limit the comparison of the results of these two studies.

Ibiapina et al.⁷ evaluated changes in transverse dimensions of upper arch and thickness of the buccal bone plate in patients undergoing orthodontic treatment with conventional and self-ligating brackets, at CEJ+3mm and CEJ+6mm. The expansion was 1.57mm and 1.77mm for interfirst premolar, and 1.08 mm and 1.88mm for intersecond premolar. They observed that a small decrease of the buccal bone occurred similarly in both groups. Our study presents an average expansion similar to Ibiapina's and the results are in agreement with that study, having found a slight decrease of only 5.2% for first PM at CEJ+4mm.

Karkhanechi et al.² report that treatments with fixed orthodontic appliances increase plaque accumulation and can interfere with oral hygiene procedures. A shift is observed from Gram+ aerobic species (associated with periodontal health) to Gram- anaerobic or facultative species (associated with periodontitis). In agreement with the study by Levrini et al.²⁷, which claims that CAT facilitates adequate oral hygiene, thereby reducing the risk of gingival-periodontal complications, our study showed that PI, GI, PPD and CAL remained stable. Regarding PI, for 1PM, 2 sites scored 1 at T0 and at T1. The same was true for 2PM. Regarding GI, for 1PM, at T0 the score was 0 at 37 sites and 2 at one site, while at T1 it was 1 at four sites. For 2PM, it was 0 at all sites at both times. Average PPD for 1PM was 1.16 at T0 and 1.32 at T1, while for 2PM it was 1.26 at T0 and 1.53 at T1, which evidences the maintenance of periodontal health. Average clinical CAL was 0.68 mm at T0 and 0.47 at T1 for 1PM, and 0.21mm at T0 and 0.11 mm at T1 for 2PM. In both groups, CAL declined, which is considered to be a clinical improvement.

With regard to the association between attachment loss and increase in distance from CEJ to CC, only one site showed positive correlation. At the sites where the CEJ-CC distance was found to increase, it could be a due to thin periodontal phenotype and the very thin initial buccal bone thickness. Nevertheless, no clinical correlation was found, possibly due to the formation of a long junctional epithelium and/or gingival contraction which prevented probe penetration.

Despite the risk of expansion in patients with a very thin buccal bone and crowding, this treatment was chosen because it provides improvements for oral hygiene and a decrease in the accumulation of bacterial plaque, guaranteeing the health of periodontal tissues for the future.

Further studies are needed to observe the behavior of periodontal tissues using larger samples and a longer follow-up period.

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CONCLUSION

Using Invisalign® for expansion movements did not produce substantial changes in the evaluated periodontal clinical parameters or in the bone measurements.

Removable appliances reduce bacterial plaque retentive factors and favor adequate oral hygiene.

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Effect of preheating on cytotoxicity and physicochemical properties of light-cured calcium-based cements

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ABSTRACT

The aim of this study was to evaluate the degree of conversion, cytotoxicity, solubility and pH of photopolymerizable calcium-based cements submitted to preheating. The degree of conversion was analyzed by Fourier transform infrared, cytotoxicity by the MTT test and solubility through loss of mass. The data were subjected to statistical tests (ANOVA / Tukey's, $p < 0.05$). The photopolymerizable materials showed a low degree of conversion, regardless of preheating. All materials caused a reduction in cell viability at 24 hours and 7 days, with the Dycal

(control) being more cytotoxic. Heat had a positive effect on Biocal at 7 days. Dycal is the most soluble material. Heat had no effect on the solubility or pH of the polymerizable materials. It is concluded that photopolymerizable calcium-based cements have a low degree of conversion and are soluble, which results in mild to moderate cytotoxicity.

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Keywords: dental pulp capping- dental cements-calcium hydroxide- polymerization- cell survival.

Efeito do pré-aquecimento na citotoxicidade e propriedades físico-químicas de cimentos à base de cálcio fotopolimerizáveis

RESUMO

O objetivo do presente estudo foi avaliar o grau de conversão, citotoxicidade, solubilidade e pH de cimentos à base de cálcio fotopolimerizáveis submetidos a pré-aquecimento. O grau de conversão foi analisado por espectroscopia no infravermelho com transformada de Fourier, a citotoxicidade pelo teste de MTT e a solubilidade através da perda de massa. Os dados foram submetidos a testes estatísticos (ANOVA/Tukey, $p < 0,05$). Os materiais fotopolimerizáveis apresentaram baixo grau de conversão, independente do pré-aquecimento. Todos os materiais causaram redução da viabilidade celular nas análises

de 24 horas e 7 dias, sendo que o Dycal (controle) apresentou-se mais citotóxico e o calor apresentou efeito positivo sobre o Biocal na análise de 7 dias. O Dycal é o material mais solúvel e o calor não causou efeito na solubilidade e pH dos materiais polimerizáveis. Assim, conclui-se que os cimentos à base de cálcio fotopolimerizáveis apresentam baixo grau de conversão e são solúveis, que resulta em citotoxicidade suave e moderada.

Palavras-chave: capeadores pulpaes- cimentos dentários- hidróxido de cálcio- polimerização- sobrevivência celular.

INTRODUCTION

Deep and very deep cavities resulting from tooth decay or trauma often require protection of the dentin-pulp complex¹. Calcium hydroxide has been the most intensively studied biomaterial over time and provided favorable clinical results², although, according to a systematic review and meta-analysis, its use is reported as unnecessary in deep caries lesions in primary and permanent teeth and has questionable clinical success. Randomized,

controlled clinical trials are still needed to provide strong recommendations on the actual need for use of calcium hydroxide³.

Calcium hydroxide remains the most commonly used pulp protection material due to its alkalinity, biocompatibility, ability to decrease bacterial infection and induce pulp tissue to form a mineralized barrier, blocking the exposed surface^{1,2,4}. However, it has some limitations, such as high solubility, poor bonding to dentin and mechanical instability⁵.

Photopolymerizable protective materials are useful because they provide greater mechanical resistance, better handling, more precise application, are minimally affected by phosphoric acid and have better marginal sealing due to their lower solubility^{6,7}. However, they have incomplete polymerization, with monomers remaining free⁷, which can cause toxic reactions to the pulp cells⁸. The biocompatibility and cytotoxicity of pulp protection materials is of paramount importance in preventing or limiting irritation to the pulp, and they have been widely studied in different cell cultures or in deep cavities, with or without pulp exposure⁹. The degree of conversion (DC) of the monomers is a measure of the percentage of carbon double bonds that have been converted to single carbon bonds to form a polymer chain resulting from the polymerization process. On average, 70% of the monomers are converted into polymers and 30% are not polymerized, with the majority of these unconverted monomers remaining in the polymer matrix, while 9% of them remain free and are able to diffuse through the dentinal tubules and reach the pulp⁷.

Some techniques to improve polymerization have been researched and it is known that preheating resin composites increases their conversion. A study by Daronch et al. (2005) used a device that heats composite resin before use, which increased monomer conversion without increasing intrapulp temperature when compared to composites placed at room temperature, showing that the preheated composite can be used safely¹⁰. In order to increase the conversion of monomers, several studies have used different polymerization methods, reporting improvements in the physical and biological properties of heated resinous materials¹¹⁻¹³.

Studies have shown that resinous materials are cytotoxic, with harmful effects on the pulp, but further studies are needed on the use of photopolymerizable calcium-based cements. Thus, our aim is to evaluate the degree of conversion, cytotoxicity, solubility and pH of photopolymerizable calcium-based cements and their effect when subjected to preheating.

MATERIALS AND METHODS

In the present study, four materials were used to protect the dentin-pulp complex, three based on calcium hydroxide: Dycal® (Dentsply Sirona, Milford, DE, USA), Biocal® (Biodynamics, Ibioporã, PR, Brazil) and Ultra-blend® plus (Ultradent

Products Inc., South Jordan, UT, USA), and one based on calcium silicate, TheraCal® LC (Bisco, Schaumburg, IL, USA).

Specimens were prepared at room temperature following the manufacturers' guidelines and photoactivated for 40 s using a device with a light-emitting diode (LED) with power adjustment at 1000 mW/cm² (VALO, Ultradent, Salt Lake City, Utah, USA). The same materials were also prepared with previous heating by placing the molds on a heated base at 39 °C in a HotSet® device (Technolife, Joinville, SC, Brazil) for 1 min, followed by photoactivation for 40 s. After photoactivation, the specimens were removed from the molds and sterilized in ethylene oxide (Esterilipus, Porto Alegre, RS, Brazil). Table 1 summarizes the experimental groups evaluated. The materials were subjected to the tests described below.

Cell viability

Specimens were prepared in a metallic mold 6 mm in diameter and 1 mm in depth (n=5) for the test, which was carried out according to ISO 10993-12: 2012¹⁴, in a similar way to that described by Portella *et al.*¹⁵

The cells used for the present study were mouse fibroblasts of the NIH 3T3 strain (ATCC® American Type Culture Collection - TCC, Old Town, Maryland, USA). Cell viability was assessed by analyzing mitochondrial activity using the methyl tetrazolium (MTT) technique. The percentage of unviable cells was calculated in relation to the negative control (cells cultured in DMEM medium).

Degree of Conversion (DC)

The DC was evaluated using the Fourier Transformed Infrared Spectroscopy (FTIR) technique. The spectrophotometer (VERTEX 70, Bruker Optics, Ettlingen, Germany) was coupled to the ATR device, composed of a 2 mm horizontal diamond crystal (Platinum ATR-QL, Bruker Optics, Ettlingen, Germany), forming an angle of 45° with mirror¹⁶. The samples (n=3) were made from a standardized volume of material that was dispensed on the crystal of the attenuated total reflectance device (ATR), being retained within a cylindrical mold 5 mm in diameter by 1 mm in depth. In the groups where the materials were heated, the material compules were heated until they reached a temperature of 39 °C using the HotSet® heating device. After being removed from the heating device, they were immediately dispensed in the mold and photoactivated for 40 s.

Table 1. Experimental groups and procedures used.

GROUP	MATERIAL	PROCEDURES
DYC	Dycal®	Room temperature + Chemical activation
BIO	Biocal®	Room temperature + Photoactivation 40'
BIO 39°C	Biocal®	39 °C + Photoactivation 40'
THE	TheraCal LC®	Room temperature + Photoactivation 40'
THE 39°C	TheraCal LC®	39 °C + Photoactivation 40'
ULT	Ultra-Blend plus®	Room temperature + Photoactivation 40'
ULT 39°C	Ultra-Blend plus®	39 °C + Photoactivation 40'

The degree of conversion was determined from the ratio of the absorbance peak corresponding to the carbon-carbon aliphatic double bond (1640 cm^{-1}) with that of the internal standard (1610 cm^{-1} for the Biocal® and TheraCal® LC materials and 1720 cm^{-1} for the Ultra-Blend® plus material). Percentage DC was calculated according to a previous study¹⁶.

Solubility and pH

Degradation was evaluated by weight loss (WL) of materials after immersing the samples in 10 mL of distilled water for 24 h and 7 days at 36°C. The dry mass of the samples was determined before (W₀) and after immersion (W_f). Average WL of the samples (n = 3) was obtained using the equation:

$$WL(\%) = (W_f - W_0) / W_0 \times 100$$

A digital pH meter (pH meter DM-23, Digicrom Analytical Ltda, São Paulo, SP, Brazil) was used to measure the pH of the storage water after 24 h and 7 days. The initial pH of the water used for the immersion of the samples was 5.96 ± 0.54 .

Statistical Analysis

The mean values determined for the experimental groups in the cytotoxicity, solubility and pH tests were compared using the two-way ANOVA test (experimental group and time), followed by the Tukey's test. For the DC test, two-way ANOVA (material and heating) and the Tukey's test were used. All analyses were performed with a 5% significance level.

RESULTS

The results show that all cements, regardless of the treatment used in the present study (chemically activated polymerization, photoactivation and heating prior to photoactivation), according to ISO 10993-5: 2009 (E)¹⁷, presented mild to moderate

cytotoxicity, demonstrated by the reduction in the number of viable cells when compared to the negative control group ($p < 0.05$) (Fig. 1). The chemically activated cement, on the other hand, presented severe cytotoxicity, statistically higher than in photopolymerizable calcium-based cements ($p < 0.05$). In the 24 h cell viability analyses, there was no statistical difference between the photopolymerizable calcium-based cements used and the preheating of the cements did not reduce their cytotoxicity. However, there is less significant cytotoxicity in light-cured calcium-based cements than in the negative control group and the DYC group. When the cell viability of 7 days was analyzed, the BIO 39 °C group showed a significant decrease in the cytotoxicity of the material, and the preheating caused a positive effect on cell viability. On the other hand, the DYC was statistically more cytotoxic than the experimental groups. When comparing the 24 h and 7 day analyses, the BIO 39 °C material showed a decrease in cytotoxicity over the extraction time of the material, while the THE and ULT materials showed a significant decrease in viability over time. The heat treatment did not result in a significant increase in the DC of the materials used. Fig. 2 shows that degree of conversion percentage for the THE material was extremely low both at room temperature (12.65 ± 10.31) and heated (7.84 ± 9.46). At room temperature, DC was significantly higher for ULT than for THE (51.87 ± 4.35 ; $p < 0.05$), while BIO (34.47 ± 21.72) did not differ statistically from either of the other products. When the materials were heated, ULT 39°C (46.30 ± 1.38) and BIO 39°C (45.71 ± 6.76) had significantly higher polymeric conversion ($p > 0.05$) than THE 39°C (7.84 ± 9.46). All materials lost 0.04% to 17.68% of their mass (Table 2). DYC immersed in distilled water for 7 days had the highest solubility among all groups, with

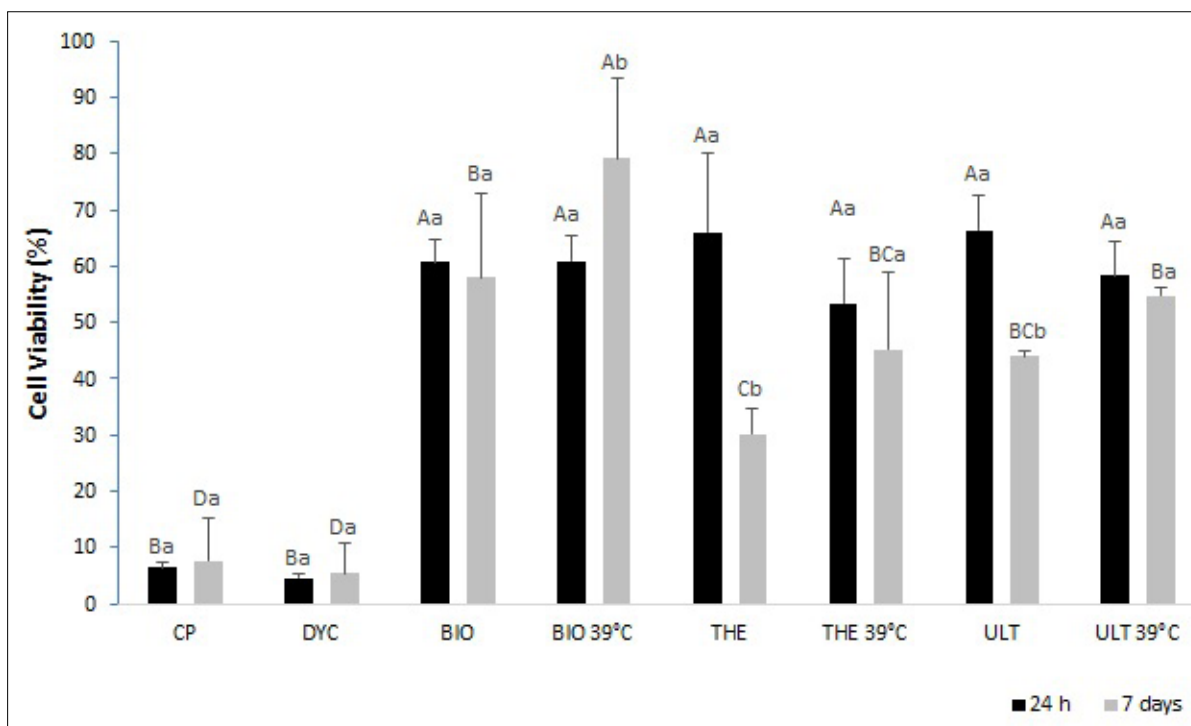


Fig. 1: Percentage of cell viability of experimental and control groups at 24 hours and 7 days. Different capital letters indicate difference between the experimental groups and different lowercase letters indicate difference in the analysis at 24 hours and 7 days.

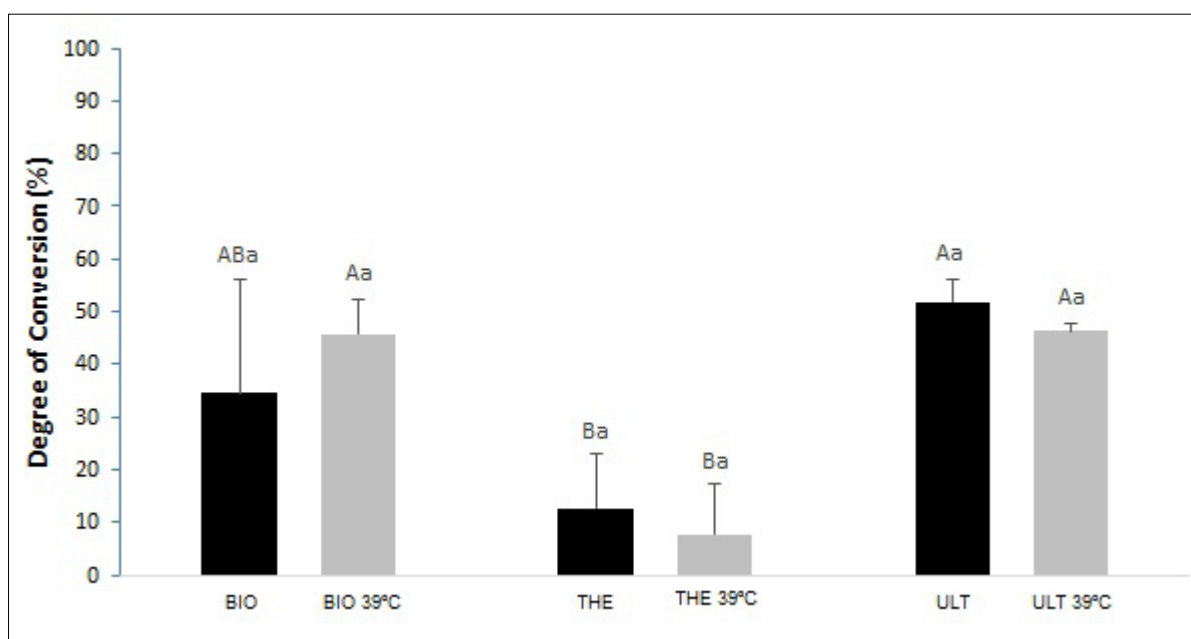


Fig. 2: Percentage of the degree of conversion of the groups at room temperature and preheated to 39 °C. Different capital letters indicate difference between materials and different lowercase letters indicate difference between groups at room temperature and preheated to 39 °C.

significant loss between 24 h and 7 days. Ultra-Blend plus® also lost significant mass over the same time. In contrast, Biocal® lost significantly less mass, with no statistical difference from TheraCal® LC at 7 days. Heat treatment had no influence on the mass loss of the materials. Table 2 also shows the pH variation of the samples analyzed at 24 h and 7 days. The medium was alkalized for all materials, with DYC and THE 39° being significantly the most alkaline.

DISCUSSION

Resin-modified calcium hydroxide or calcium silicate cements are promising dental products for pulp protection⁷. However, it is believed that the release of unpolymerized monomers has great impact on cytotoxicity in pulp cells by both direct and indirect contact, by diffusion of the monomers through the transdental route to the pulp tissue^{12,13,18}. In addition to decreasing cell viability, this causes the release of pro-inflammatory cytokines and recruitment of inflammatory cells, thus compromising the regenerative process¹⁹.

Considering that these materials are used in close proximity to the pulp, they should have the ability to protect it in addition to having a high degree of conversion to reduce the possibility of release and diffusion of monomers that can cause pulp damage²⁰. However, the degree of conversion was found to be lower than expected for all materials, with half or less than half of the monomers converted into polymers, even when the photoactivation time employed was double that recommended by the manufacturer. The results of the present study differ from the study by Soares *et al.*²¹ where ULT conversion was close to 90% and the BIO showed a significantly lower DC

than all other leveling materials, close to 75%.

According to some authors^{22,23}, the release of unpolymerized monomers from resin composites is greatest during the first 24 h. However, there is a continuous release over time at a reduced rate, due to hydrolysis and degradation of resin-based materials²²⁻²⁶. It is believed that the longer the extraction time, the greater will be the amount of unconverted monomers leached from the resin matrix into the extraction medium. Arias-Moliz *et al.*²⁷ reported that over time, some cements showed an increase in cytotoxicity, and THE became more cytotoxic in 7 days. This is in agreement with our study and may be related to its low percentage of DC. Thus, we noticed that all the tested materials increased the cytotoxicity of the analysis from 24 hours to 7 days, except the BIO 39°C, suggesting that the increase in temperature may have caused greater conversion of the monomers, resulting in an improvement in the biological properties of the product. This relates to our findings that the BIO 39 °C achieved a higher degree of conversion when heated than when only photoactivated, even though the difference was not statistically significant. In the 7-day analysis, BIO 39°C showed no statistical difference when it was only photoactivated, but it showed less cytotoxicity than the other materials tested.

Exposing materials to high temperatures – within a degree biologically compatible with the pulp – has significant effects on their polymerization, increasing the DC^{28,29} and consequently decreasing their cytotoxicity, as demonstrated by Klein-Júnior *et al.*¹², who reported a reduction in cytotoxicity at 24 h and at 7 days in self-adhesive resin cements

Table 2. Solubility and pH in the experimental groups according to time.

	Weight loss (%)		pH	
	24 hours	7 days	24 hours	7 days
DYC	2.98±0.16BCb	17.68±5.39Aa	10.62±0.09Ab	11.77±0.02Aa
BIO	0.13±0.13Ca	0.52±0.78Ca	7.40±0.42Cb	10.11±0.01Ca
BIO 39°C	0.04±0.07Ca	0.13±0.18Ca	7.62±0.22BCb	9.79±0.18Ca
THE	2.27±0.16BCa	2.29±1.15Ca	8.08±0.02BCb	10.98±0.29Ba
THE 39°C	2.46±1.32BCa	1.31±1.64Ca	10.88±0.13Aa	11.02±0.04Aa
ULT	5.05±0.2Bb	11.87±0.55Ba	7.42±0.11Ca	7.70±0.43Da
ULT 39°C	4.03±2.17Bb	11.26±1.00Ba	7.31±0.04Cb	8.22±0.20Da

Different capital letters indicate difference between the experimental groups and different lowercase letters indicate difference in the analysis at 24 h and 7 days.

heated to 60 °C. It is believed that heating promotes greater molecular mobility, increasing the frequency of collision of reactive species and postponing the spread of controlled diffusion (auto deceleration), thus resulting in a highly reticulated network of polymers, and presenting a better conversion of monomers²⁹.

In contrast, we did not have significant results with preheating, in agreement with Fróes-Salgado *et al.*³⁰, who reported that pre-heating composite resin did not change its degree of conversion, although it provided better marginal adaptation to the cavity walls. In addition to the temperature of the material being slightly below that supplied by the heating unit, there is a 50% drop in temperature 2 min after the material is removed from the heating unit²⁹. The time possibly elapsed between the removal of material from the device and its photoactivation may have influenced the DC of the material. Another hypothesis would be that the temperature of 39 °C is not sufficient to promote a significant increase in monomer conversion.

The DC results for the analyzed materials were relatively low, considering the average value of composites is about 70%⁷. As previously reported by other authors, heat should have improved the DC of these materials¹¹. Again, the question of the calcium hydroxide component and its solidity, which becomes a barrier to the mobility of monomers, is raised. It is important to understand that the chemical components of calcium hydroxide, radiopacifiers and derivatives may hinder the conversion of these monomers and prevent heat from further improving this conversion.

Both the chemical composition of the resinous material and the percentage of conversion of monomers in dental materials are considered as a potential cause of cytotoxicity²⁵. In a previous study, Deb *et al.*³¹ showed that preheating at 60 °C had no effect on cytotoxicity, attributing the different levels of cytotoxicity of the materials to their compositions. The monomers present in resin composites and adhesive systems (e.g., BisGMA, UDMA, TEGDMA, HEMA) have already been shown to have cytotoxic effects in direct contact with fibroblasts and can be leached when the DC is not fully attained⁹. Compounds such as bisphenol glycidyl methacrylate (BisGMA), urethane dimethacrylate (UDMA) and camphorquinone can induce major cell damage³². In 2003, Chen *et*

*al.*³³ showed that the BisGMA monomer is the one with the highest cytotoxicity, followed mainly by the UDMA, TEGDMA and HEMA monomers, and verified that interactions between these monomers enhance the cytotoxicity of each one. In the study by Schwengberg *et al.*³², BisGMA exposure resulted in a 50% decrease in cells, and in the study by Ratanasathien *et al.*³⁴, the UDMA proved to be less cytotoxic than the BisGMA after 24 hours and 72 hours of exposure, which may explain the cytotoxic effect found for TheraCal® LC in the present study. Lee *et al.*³⁵ attributed to the BisGMA found in this material an extensive pulp inflammatory response with a 75% decrease in cell viability. Similarly, Nilsen *et al.*³⁶ reported a higher concentration of camphorquinone in this material than in other protective materials based on resin.

There are several previous studies on the cytotoxicity of pulp protection materials^{37,38}, with the Dycal® showing the greatest cytotoxic effects, even when compared to pulp protection cements. Hirschman *et al.*³⁷ reported that the cytotoxic effect of Dycal® was greater than that of Ultra-Blend® plus, in agreement with our results. When testing light-cured calcium-based cements, we found a mild and moderate degree of cell cytotoxic reactivity according to the ISO 10993-5: 2009¹⁷ standards, and in terms of biocompatibility they are superior to Dycal®, which caused severe cell cytotoxicity.

Even with high initial cytotoxicity, the alkaline medium is important to promote cell differentiation, inducing the formation of secondary dentin, as well as the formation of the mineralized barrier in cases of pulp exposure³⁸. On the other hand, Luczaj-Cepowicz *et al.*³⁹ reported that alkaline pH most likely does not affect the viability of cells in culture. In the present study, Dycal® had the most alkaline pH, the greatest solubility in the 7-day analysis and the greatest toxic effect on cells in culture.

The solubility of the material has an antagonistic character. There is a need for a certain solubilization of calcium ions for the alkalization of the medium to occur and, consequently, tissue repair⁹. However, when this loss of mass is very significant, an empty space remains between the mineralized tissue and the restorative material. Thus, there is a need to achieve a balance between being soluble enough to have a therapeutic effect on the pulp tissue and not compromising the restorative procedure mechanically⁴⁰.

In conclusion, light-cured calcium-based cements have low DC, mild to moderate cytotoxicity and varying solubility. Preheating the material showed no effect on DC, solubility or cytotoxicity, except for

BIO 39°C at 7 days. Chemically activated calcium hydroxide cement was more soluble and cytotoxic than polymerizable materials.

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Dental caries in three-year-old preschool children in Lima, Peru assessed according to the CAST instrument

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ABSTRACT

The aim of this study was to determine the caries situation of three-year-old preschool children residing in low socio-economic status districts in Lima, Peru. The study is a cross-sectional analysis of the caries situation of suburban areas of Lima. A stratified sampling procedure by geographical distribution, considering healthcare centers with a mother-and-child health clinic and surrounding preschools as factors, identified 45 randomly selected preschools, of which 17 accepted to participate. Children from 3-year-old classrooms were examined by two independent calibrated dentists using the Caries Assessment Spectrum and Treatment (CAST) instrument at their premises using artificial light, sterile examination mirrors and gauze for drying each tooth before evaluation. ANOVA and the Tamhane method were used to analyze the

data. 308 children, mean age 3.4 years (min: 3 years; max: 3 years, 7 months), were examined. The sample prevalence of enamel and dentine carious lesions (CAST code 3-7) was 91.2% while the prevalence of dentine carious lesions (CAST code 4-7) was 58.8%. The mean number of teeth with cavities that had reached the pulp and those that had an abscess or fistula were 2.0% and 0.5% respectively. The majority of enamel and dentine carious lesions were observed in molars. The CAST severity score was 7.0. Mean examination time was 57 seconds. The burden of dental caries of the children at this young age was high.

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Keywords: dental caries- cross sectional studies- epidemiology- public health dentistry- child, preschool.

Caries dental en niños preescolares de 3 años en Lima-Perú evaluados con el instrumento CAST

RESUMEN

El objetivo del presente estudio fue determinar la prevalencia de caries dental en niños en edad preescolar de 3 años residentes en áreas suburbanas de Lima, Perú. Se trata de un análisis transversal de la situación de caries de áreas periféricas de Lima. Un procedimiento de muestreo estratificado por distribución geográfica consideraba a los centros de salud materno-infantiles y centros educativos preescolares de la jurisdicción como factores, identificando 45 centros preescolares aleatoriamente, de los cuales 17 aceptaron la invitación para participar del presente estudio. Dos odontólogas independientes, calibradas examinaron a los niños de las aulas de 3 años utilizando el instrumento Caries Assessment Spectrum and Treatment (CAST) en las instalaciones de cada jardín de infancia, utilizando luz artificial, instrumental estéril y gasas para el secado de las superficies a evaluar. Los datos fueron analizados utilizando ANOVA y el método Tamhane. Se

evaluaron 308 niños, quienes tenían una edad media de 3.4 años (min: 3 años; max: 3 años, 7 meses). La prevalencia de lesiones de caries de esmalte y dentina (código CAST 3-7) fue del 91,2%, mientras que la prevalencia de lesiones de caries en dentina (código CAST 4-7) fue de 58,8%. El número promedio de dientes afectados por caries dental con compromiso pulpar y que tenían un absceso o fístula fue de 2.0% y 0.5% respectivamente. La mayoría de las lesiones de caries en esmalte y dentina se observaron en los molares. La valoración de severidad CAST fue 7.0. El tiempo promedio de examinación fue de 57 segundos. La carga de la enfermedad caries dental a estas edades tan tempranas ya es alta en la infancia suburbana de Lima.

Palabras clave: caries dental- estudios de corte transversal- epidemiología- salud pública odontológica- niños, preescolares.

INTRODUCTION

Despite being preventable, dental caries is the main public health problem worldwide and its consequences are often untreated¹. The clinical signs manifest initially as enamel white-colored lesions, which may develop into cavitated dentine lesions. The progression of a carious lesion is an unwanted situation that could affect a child's normal development during early years of life by impeding proper nutrition, proper sleep and the development of self-esteem^{2,3}. Untreated dentine carious lesions may even result in pain and suffering, and may lead to pulp involvement, abscesses, fistula and early extraction of these recently emerged primary teeth⁴. Low socio-economic status (SES) and limited education of mothers have been reported as major risk factors for the development of carious lesions in children⁵. High-caries-risk mothers and their children have a tendency to visit the dental practitioner only when the consequences of the disease are evident. To avoid this situation, the dental practitioner has to consider ways to interact with parents as from the time their children's teeth erupt. The obvious place where this should happen is at the mother-and-child health clinics at healthcare centers. As dental personnel are not usually employed at these centers, they should actively seek involvement with center health professionals⁶. Nurses appear to be the most appropriate health professionals to engage with because they see parents on a regular basis as part of their children's vaccination, growth and development program. But in order for nurses to be effective in improving the oral health of infants, they need to be educated in oral health promotion and preventive measures⁷.

To measure the impact of nurses in a primary oral healthcare program, reference epidemiological data are needed. A sensitive diagnostic instrument is needed for examining very young children in a population with expected extensive caries situation. The instrument should cover the full spectrum of dental caries, including enamel carious lesions and those with extensive dental pathology. This requirement excludes the WHO criterion, which only considers presence or absence of a dentine cavity. Moreover, the International Caries Detection and Assessment System (ICDAS) is not complete, because it does not record extended dental pathology into the pulp and mucosal tissue⁸. The caries assessment tool that appears to meet these

requirements is the recently developed and validated Caries Assessment Spectrum and Treatment (CAST) instrument. CAST uses the epidemiological concept of health and disease and considers treated surfaces with sealants and restorations as healthy. The CAST codes are hierarchically ordered from no carious lesion through a carious lesion in enamel and in dentine to pulp-involved and abscessed teeth, and teeth missing due to dental caries⁹. The present study is the first to present the results of a caries epidemiological survey that used the CAST instrument as recommended in the CAST manual¹⁰ and used its severity score¹¹.

Information on the prevalence and severity of dental caries amongst youth in Lima is scarce, outdated and published in Spanish. To counter this paucity of information and as dental caries reference data are required to monitor carious lesion development across the younger sector of the population in the future, the present study was conducted with the aim of reporting the caries situation of three-year-old preschool children residing in three low SES districts.

MATERIALS AND METHODS

Ethical approval

Ethical approval for the study was obtained from the Institutional Review Board of the Dental School of San Martin de Porres University (Lima-Peru) (Resolution No. 252-2013-D-FO-USMP). This cross-sectional study is related to a randomized clinical trial (RCT) that is registered in the Netherlands Trial Centre with number NTR 4510¹². Parents received a letter with general information about the epidemiological survey and an informed consent form. They were requested to sign the form and return it to the school authorities. Only children whose parents had signed and returned the form were included in the study. Every parent received a short report on their child's oral condition and suggestions for treatment, and was advised to contact the health center dentist. The research was conducted in full accordance with the World Medical Association Declaration of Helsinki.

Study design

The sampling units for the present survey were healthcare centers with well-functioning district mother-and-child health clinics, located in three districts from low socio-economical stratum (SES)

areas in Lima, Peru. The selection was guided by economic indicators used by the Peruvian National Institute of Statistics and Informatics¹³. From a total 10 eligible districts, 3 were randomly selected (AG=district 1, PG = district 2 and CG= district 3). In each of the three selected districts, there was a healthcare center with a well-functioning mother-and-child health clinic.

On the basis of being situated within 2 km radius from the healthcare center, a total 45 government preschools were randomly selected to participate, of which 17 agreed to take part in the present survey. Three-year-olds from these schools who had visited the mother-and-child vaccination clinics and growth and development program at the selected health canters from birth were invited to participate in the current survey. Invitation letters were sent to the parents.

Caries assessment

Caries assessment was conducted by two pediatric dentists who were experienced in performing epidemiological surveys among young children in a field situation. Prior to the examinations, the dentists were trained to use the CAST instrument and calibrated under the guidance of a senior epidemiologist (JEF). The training involved a theoretical explanation of the CAST codes, their descriptions and background information and a practical session in applying CAST to 20 extracted teeth, each mounted in an acrylic base. The examiners' scores were compared and differences discussed until consensus was reached. One week after the training, the examiners visited a school that was situated far away from the three study districts and which conformed to the same socio-economic profile as the survey schools. The examiners and a senior researcher (RV) examined 20 4-year-old children and compared and discussed the findings until they were all in agreement. The examiners examined eight children one week later to conduct the examiner agreement tests. The kappa-coefficient values for inter- and intra-rater reliability for the two examiners were 0.75 and 0.81, and 0.74 and 0.75, respectively. These results were considered sufficiently high to begin the epidemiological survey.

The oral examinations were performed at the preschool facilities. Prior to examination, patients' teeth were cleaned with a toothbrush, toothpaste

and floss (when needed) by the first author. The child lay on a cushioned table with plastic cover and the examinations were performed using sterile instruments, gauze and a battery-powered headlamp (Energizer 3 LED headlamp, Energizer Holdings Inc. USA). All surfaces of all teeth were assessed according to the CAST instrument (Table 1). The CPI probe was used to remove any plaque that was left behind after tooth brushing. Each tooth was dried with gauze. As they made the oral examination, the examiner's commentary was recorded on a digital device and transcribed on an Excel sheet. The device measured examination time.

Disposition of subjects

A total 450 children were approached. Of these, the parents of 308 children signed the consent form and those children were consequently examined.

Statistical analysis

All children from the 17 preschools who fulfilled the inclusion criteria and were present on the examination day were examined. Data were analyzed by an experienced statistician using the statistical package SPSS version 24.0 (IBM, Armonk NY, USA). CAST codes 0-2 and code 8 are excluded from the calculation of the prevalence of dental caries as they are not considered to reflect a diseased situation. The prevalence of dentine carious lesions (dental caries) is calculated using CAST codes 4-7. CAST codes 3-7 are used for calculating the prevalence of enamel and dentine carious lesions combined. Reporting the dental caries situation using the CAST instrument includes the maximum (max) CAST code per tooth (the highest code among the codes of all surfaces on an examined tooth); the max CAST code per subject (the highest code among the codes of all teeth examined in a subject); and the CAST severity score, which is obtained by first selecting the max CAST code per tooth and applying it to Formula F1, as shown below¹¹:

$$F1 = 0.25 * \text{CAST3} + 1 * \text{CAST4} + 2 * \text{CAST5} + 4 * \text{CAST6} + 5 * \text{CAST7} + 6 * \text{CAST8}$$

The 'number*' denotes the weight given to the accompanied CAST code. CAST codes 0, 1 and 2 are not part of this formula since they indicate healthy condition. CAST scores 3-8 make up the formula and are assigned weights that increase with the

Table 1. Codes and descriptions of the hierarchically ordered CAST epidemiological instrument, including disease status

Characteristic	Disease status	CASTcode	Description
Sound	Healthy	0	No visible evidence of a distinct carious lesion is present
Sealant	Healthy	1	Pits and/or fissures are at least partially covered with a sealant material
Restoration	Healthy	2	A cavity is restored with an (in)direct restorative material
Enamel	(Pre)morbidity	3	Distinct visual change in enamel only. A clear caries related discolouration is visible, with or without localised enamel breakdown
Dentine	Morbidity	4	Internal caries-related discolouration in dentine. The discoloured dentine is visible through enamel which may or may not exhibit a visible localised breakdown of enamel
	Morbidity	5	Distinct cavitation into dentine. The pulp chamber is intact
Pulp	Severe Morbidity	6	Involvement of pulp chamber. Distinct cavitation reaching the pulp chamber or only root fragments are present
Abscess/Fistula	Severe Morbidity	7	A pus containing swelling or a pus releasing sinus tract related to a tooth with pulpal involvement
Lost	Mortality	8	The tooth has been removed because of dental caries
Other		9	Does not correspond to any of the other descriptions

increase in the CAST codes: an increase in severity. The maximum weight is assigned to CAST 8, which indicates tooth loss (mortality). Consequently, the higher the final CAST severity score, the worse the carious condition of the tooth or dentition¹¹. A cumulative MaxCASTsubject is calculated using the highest CAST code for a person. Mean dmft/DMF scores can be calculated using CAST for comparison with studies done in the past¹⁴.

The CAST severity scores were compared among the three groups using ANOVA. Post hoc analyses were undertaken according to the method of Tamhane, which is suitable if variances between groups are different. Significance level was set at $p < 0.05$.

RESULTS

The sample consisted of 55.8% girls and 44.2% boys. Children's mean age was 3.4 years (min: 3 years; max: 3 years, 7 months). Mean time to examine the children was 57 seconds (range 34-130 seconds).

Prevalence of carious lesions

For the whole sample, prevalence was 58.8% for dentine carious lesions (CAST codes 4-7) and 91.2% for enamel and dentine carious lesions (CAST codes 3-7 combined). These values did not differ significantly across intervention groups (Table 2). CAST severity score for the sample was 7.0 with confidence limits of 6.0 to 7.9. It was significantly

higher for PG (9.8) than for AG (5.5) ($p = 0.003$) and CG (5.9) ($p = 0.011$). Mean dmft score was 2.6 with SD 3.2.

Distribution of CAST codes by teeth

All but one child had 20 teeth. In the sample, sound teeth were seen most often (66.0%), followed by teeth with enamel carious lesions (20.8%) and teeth that had a restorable dentine carious lesion (9.2%). The proportion of teeth with cavities that had reached the pulp and those that had an abscess/fistula was 2.0% and 0.5% respectively. The majority of enamel and dentine carious lesions were observed in molar teeth, followed by incisors. The prevalence of dentine carious lesions in incisors, cuspids, 1st and 2nd molars was 8.9%, 3.1%, 25.9% and 25.6%, respectively.

Distribution of CAST codes per child

The frequency distribution of the maximum CAST code per child by sample is presented in Figure 1. Most children in the sample had a restorable dentine carious lesion as the highest code (35.4%), followed by a tooth with an enamel carious lesion (32.5%). An unexpectedly high percentage of children had cavities that had reached the pulp (15.6%). This phenomenon was most prevalent in children from the PG group. Figure 2 shows the frequency distribution of enamel lesions and dentine lesions (cavitated) in the three districts included in the study.

Table 2. Carious lesion prevalence (%), CAST severity scores and mean dmft-scores by group and sample

Caries prevalence (%)	Districts included			Sample
	AG	PG	CG	
All carious lesions CAST codes (3-7)	91.0%	92.8%	90.0%	91.2%
Dentine lesions CAST codes (4-7)	51.4%	64.9%	61.0%	58.8%
CAST severity score	5.5 ^a	9.8 ^b	5.9 ^c	7.0
95% Confidence Interval	4.1-6.8	7.6-12.0	4.6-7.3	6.0-7.9
Mean dmft-score (SD)	2.1 (2.9)	3.4 (3.8)	2.4 (2.9)	2.6 (3.2)

AG=District 1; PG=District 2; CG=District 3

P_{a,b}=0.003; P_{b,c}=0.011

SD=Standard Deviation

The burden of dentine carious lesion was lowest in the AG group, but these children showed the highest prevalence of carious lesions in enamel.

DISCUSSION

The present survey is the first to have used the CAST instrument amongst three-year-olds, and presented the CAST severity score and reported

epidemiological results differently from the usual way. Mean dmft-scores were calculated using CAST codes but, in essence, they are not needed¹⁴. They are included in the present report to enable comparison with previous studies conducted in Peru that used the dmft index. The CAST instrument was used in a study among two- to four-year-olds in Tanzania, but the results were presented as mean

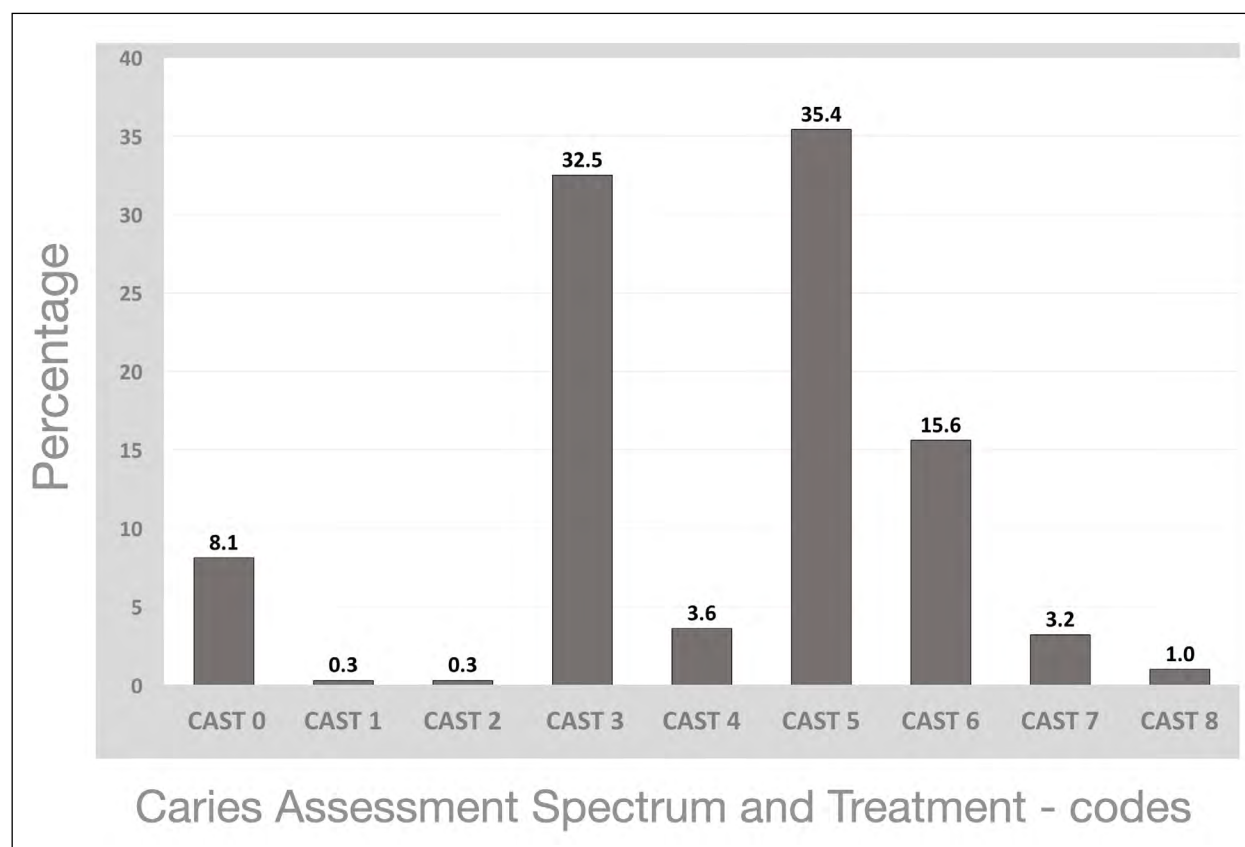


Fig.1. Frequency distribution (%) of maximum CAST code per child for the total sample of three-year-olds.

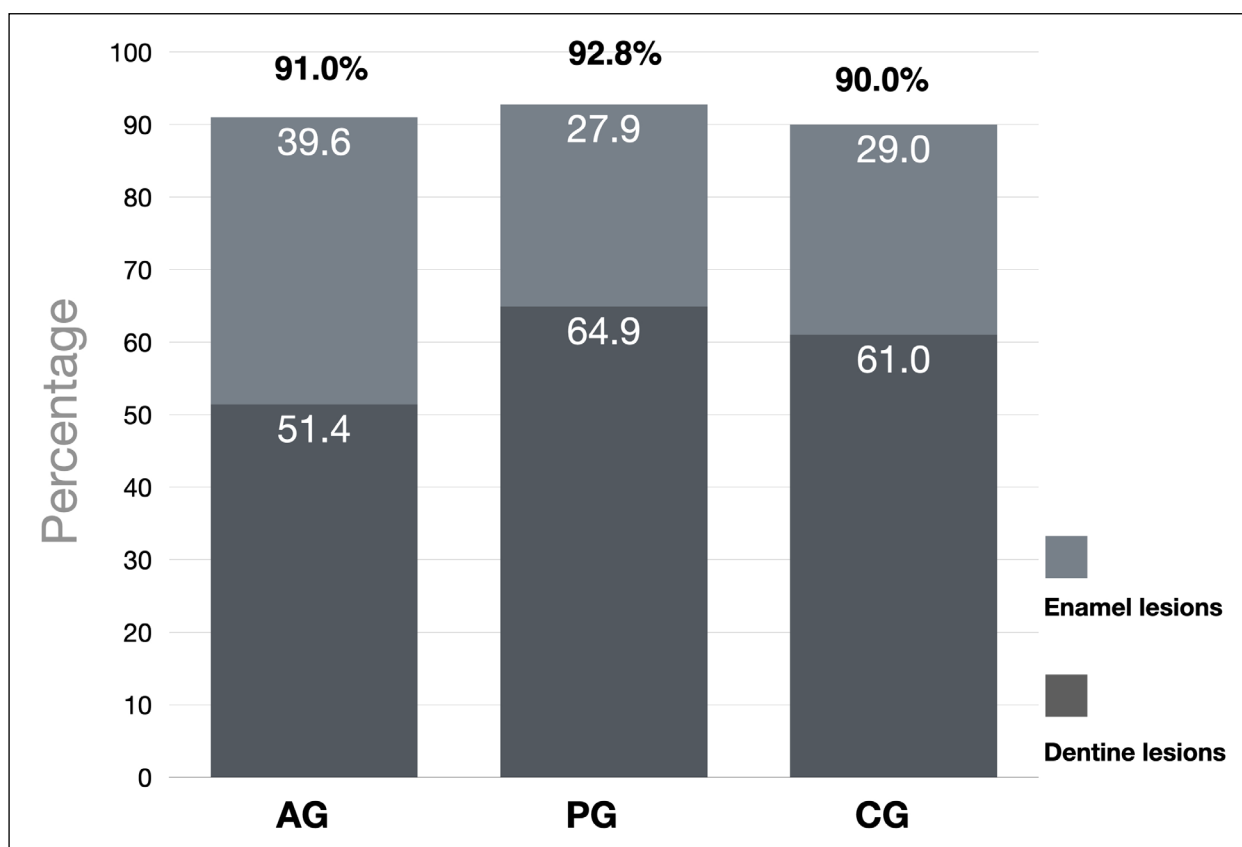


Fig. 2. Frequency distribution (%) of enamel lesions (non-cavitated) and dentine lesions (cavitated) in the three districts included in the study. AG=District 1; PG=District 2; CG=District 3.

dmft-scores only¹⁵. The dmft-index has been used over many years and is considered outdated because it mixes active disease with treated disease. Such a combination is unable to show gains in health after intervention¹⁶. The CAST instrument considers treated teeth as no longer diseased, and therefore enables the expression of improvement in the caries situation in populations over time, by showing lower dentine carious lesion prevalence and lower caries severity score.

Prevalence of dentine carious lesions in the present study was 58.8%. If enamel carious lesions are included in the disease calculation, prevalence increases to 91.2%. It can only be concluded that dental caries is highly prevalent among young children with low SES. Epidemiological studies at this early age are important because they provide data for decision-makers, who should implement preventive strategies from birth rather than waiting and having to repair the consequences of the disease. To prevent dentine cavities, sugar consumption should be reduced and oral hygiene improved,

which may require unconventional actions. These strategies should be carried out by dental health care providers at the mother-and-child health clinics in healthcare centers.

A similar high prevalence of dentine carious lesions (62.3%) was observed in a different low-SES group of Peruvian three-year-olds¹⁷. In Cambodia¹⁸ and Vietnam,¹⁹ the prevalence of dentine carious lesions among three-year-olds was also very high, at 65.6% and 74% respectively. However, the opposite has also been reported. In Nigeria, the prevalence among this age group was 1%²⁰ and in Tanzania, among two- to four-year-olds, the prevalence of dentine carious lesions was 5.3%¹⁵. The results of the latter two studies show that dentine carious lesion development can be managed.

Apart from the high prevalence of dentine carious lesions, the prevalence of enamel carious lesions was also high (42.5%). These enamel and dentine carious lesions have developed over the first 30 months of the life of these infants. During that relatively short period, 15.5% of these infants had pulp-involved

carious teeth and 3.2% of infants had an abscess. Posterior teeth were more affected by dentine carious lesions than anterior teeth. Considering that molars start emerging when the infant is 18- to 30-months old, it is clear that parents/members of these communities need to be guided in keeping erupted teeth healthy as early in the life of the infant as possible. Early childhood caries could extend its consequences to permanent dentition, generating a life-long burden of disease to the individual²¹.

Since the first publication in 2011²², the CAST instrument has been studied, applied and discussed in an ever-growing number of publications from different countries. It has the advantage over ICDAS that it also includes carious lesions with pulp involvement and abscessed teeth. CAST does not differentiate cavitated from non-cavitated enamel lesions, which might be a subject for discussion. However, as CAST is meant for use in epidemiological surveys, in contrast to ICDAS, which is also meant to be used in private practice, having one code for determining the presence of an enamel lesion is considered sufficient and realistic. Additionally, the three ICDAS codes for scoring enamel carious lesion are combined in reporting the results, which calls to question the need for three codes¹⁶. CAST makes a distinction between dentine carious lesions that can be treated restoratively and that have reached the pulp. Presenting the results as cumulative CAST codes per child, as shown in Figure 2, makes it possible to identify the level of disease in a community very easily. The longer the

line remains low, the more diseased the population is. These advantages have been documented in studies where the CAST instrument has been applied²³⁻²⁵.

Compared to the WHO caries assessment criterion, CAST took an equally short time to complete the assessment of 7- to 11-year-olds and the mean dmf/DMF scores derived from the CAST data did not differ significantly from those obtained by using the WHO criterion¹⁴. The advantage of CAST over the WHO criterion is related to the more detailed collection of data of the caries process rather than recording the presence of a dentine cavity, a restoration and an extracted tooth.

The present survey has limitations. The results are not representative of the dental caries situation of three-year-olds in Lima. Its internal and external validity is therefore low. The drop-out percentage of 31.6% can be considered substantial but is apparently not uncommon (parents who did not sign the informed consent) in children from low-SES populations⁴ and the final sample met the required sample size. Notwithstanding these limitations, the survey has provided results that should alarm the authorities and the dental profession in Lima and that necessitate a tailor-made solution.

CONCLUSION

The burden of dental caries in this group of three-year-olds is alarmingly high. This report can facilitate future decision-making regarding health policies for populations.

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Effect of two processing techniques used to manufacture lithium disilicate ceramics on the degree of conversion and microshear bond strength of resin cement

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ABSTRACT

The aim of the current study was to compare the effect of two lithium disilicate ceramic processing techniques (pressing and CAD/CAM) on the degree of conversion of resin cement when it is photoactivated and the microshear bond strength of resin cement to ceramics. Two ceramic discs were manufactured: one by the Press technique and another by CAD/CAM technique. Five Variolink Veneer resin cement samples were photoactivated through each ceramic disc and subjected to attenuated total reflectance Fourier transform infrared spectroscopy to evaluate their degree of conversion. To evaluate microshear bond strength, 20 ceramic bars were prepared: 10 using the Press technique and 10 using the CAD/CAM technique. The bars were air abraded with 50- μ m Al_2O_3 particles, treated with 10% hydrofluoric acid for 20 s and subjected to RelyX Ceramic Primer application. Adper Scotchbond Multi-Purpose Adhesive was applied to the bars and cured for 10 s. Two 1-mm-height plastic tubes were

placed on each bar and filled with Variolink Veneer resin cement. Each cylinder was cured for 20 s. Then the plastic tubes were removed and the microshear bond strength of each cylinder was tested. Data for degree of conversion (%) and microshear bond strength (MPa) were subjected to Student's t-test ($\alpha = 0.05$). Both degree of conversion of resin cement photoactivated through ceramic and microshear bond strength of resin cement to ceramic were statistically higher in the group in which ceramic was prepared by CAD/CAM technique (44.74% and 22.18 MPa) than in the group in which the ceramic was prepared by the Press technique (25.71% and 19.83 MPa). The lithium disilicate ceramic processing techniques affected the degree of conversion of resin cement when it is photoactivated through the ceramics and the microshear bond strength of resin cement to ceramics.

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Keywords: ceramics-resin cements-cementation- polymerization.

Efeito de duas técnicas de processamento utilizadas na fabricação da cerâmica de dissilicato de lítio no grau de conversão e na resistência de união ao microcissalhamento do cimento resinoso

RESUMO

O objetivo do presente estudo foi comparar o efeito de duas técnicas de processamento da cerâmica de dissilicato de lítio (prensada e CAD/CAM) no grau de conversão do cimento resinoso quando fotoativado e na resistência de união ao microcissalhamento do cimento resinoso às cerâmicas. Foram fabricados dois discos de cerâmica: um pela técnica prensada e outro pela técnica CAD/CAM. Cinco amostras de cimento resinoso Variolink Veneer foram fotoativadas através de cada disco cerâmico e submetidas à espectroscopia no infravermelho por transformada de Fourier com reflectância total para avaliar seu grau de conversão. Para avaliar a resistência de união ao microcissalhamento, foram preparadas 20 barras de cerâmicas: 10 utilizando a técnica prensada e 10 utilizando a técnica CAD/CAM. As barras foram tratadas com

partículas de Al_2O_3 (50 μ m), com ácido fluorídrico a 10% por 20 s e submetidas à aplicação do RelyX Ceramic Primer. Em seguida, o adesivo multiuso Adper Scotchbond foi aplicado nas barras e fotoativado por 10 s. Dois tubos de plástico de 1 mm de comprimento foram colocados em cada barra e preenchidos com cimento resinoso Variolink Veneer. Cada cilindro foi fotoativado por 20 s. Os tubos de plástico foram removidos e a resistência de união ao microcissalhamento de cada cilindro foi testada. Os dados de grau de conversão (%) e resistência de união ao microcissalhamento (MPa) foram submetidos ao teste t de Student ($\alpha = 0,05$). Tanto o grau de conversão do cimento resinoso fotoativado através da cerâmica quanto a resistência de união do cimento resinoso à cerâmica foram estatisticamente maiores no grupo em que a cerâmica foi confeccionada pela técnica CAD/CAM

(44,74% e 22,18 MPa) do que no grupo em que a cerâmica foi confeccionada pela técnica prensada (25,71% e 19,83 MPa). As técnicas de processamento de cerâmica dissilicada de lítio afetaram o grau de conversão do cimento resinoso quando fotoativado através das cerâmicas e a resistência

de união do microcisalhamento do cimento resinoso às cerâmicas.

Palavras-chave: cerâmica- cimentos de resina-cimentação-polimerização.

INTRODUCTION

The global demand for ideal dental aesthetics is based on the assumption that beauty is associated with favorable personality traits¹. Ceramics are used in dentistry as aesthetic restorative materials due to their satisfactory mechanical properties such as high compressive strength, chemical stability, low electrical conductivity, thermal diffusivity, translucency, fluorescence, biocompatibility (they provide low solubility, reduce plaque accumulation and enable sufficient marginal adjustment), favorable aesthetic properties and thermal expansion coefficient similar to that of tooth structure^{2,3}. However, the brittle nature of ceramics has led to high fracture rates, mainly in the case of glass-ceramic systems placed in the posterior region⁴.

Lithium disilicate glass-ceramic system is a unique material with microstructure interconnected with lithium disilicate crystals similar to needles making up to 70% volume incorporated into a glassy matrix⁵. It is used in indirect restorations to emulate the aesthetics and natural resistance of dental structures⁶. It provides translucency similar to that of natural teeth and biaxial resistance of up to 400 MPa, which is almost three times higher than that of conventional glass ceramics⁵. The use of ceramic veneers in dentistry has increased due to high degree of aesthetic requirements, since lithium disilicate facets can restore dental aesthetics by making the restoration look very similar to the natural dental structure. Moreover, it requires less invasive preparations or even no cavity preparation at all⁷. The aesthetic quality of the material is not the only criterion to be taken into consideration to ensure successful restoration procedures. Ceramic veneers must be firmly attached to the dental structure by resin cements in order to ensure good strength, aesthetics and clinical longevity^{8,9}.

According to their polymerization method, resin cements can be classified as chemically activated, physically activated or dual activation (dual cement), which combines both activation forms. Light-activated resin cements are the material of choice in situations such as cementation of

ceramic veneers due to their color stability and long working time¹⁰. However, the maximum degree of conversion (DC) of light-activated resin cement cannot be ensured in situations where light has been attenuated. Insufficient degree of conversion can adversely affect the mechanical properties, change the dimensional stability and decrease bond strength between resin cements and dental and ceramic structures, thereby impairing the clinical longevity of restorations¹¹. Accordingly, based on the aforementioned assumptions, further studies are needed to clarify the effect of processing techniques of the lithium disilicate-based restoration on the degree of conversion and bond strength of resin cement. The aim of the current study was thus to compare the effect of two lithium disilicate ceramic processing techniques (pressing and CAD/CAM [Computer Aided Design/Computer Aided Machining]) on the degree of conversion of resin cement when it is photoactivated and the microshear bond strength of resin cement to ceramics. The null hypotheses are that there is no difference between the two processing techniques regarding either [1] the degree of conversion of resin cement or [2] the microshear bond strength of resin cement to ceramics.

MATERIALS AND METHODS

Ceramic disc and bar Preparation

Two discs (10 mm diameter by 0.6 mm thick) and twenty bars (6.5-mm long x 5-mm wide x 1-mm thick) were manufactured in lithium disilicate glass ceramic. One disc and 10 bars were based on the manufacturing technique applied to lithium disilicate ceramic indirect restorations, followed by the automation technique guided by the CAD/CAM system (IPS e.max CAD; Ivoclar Vivadent, Schaan, Liechtenstein), while the other disc and 10 bars were prepared by isostatic injection based on heat technology (IPS e.max Press; Ivoclar Vivadent), both in shade LT A1. The resulting discs were subjected to sequential wet polishing with silicon carbide abrasive papers (320, 400, 600 and 1200

grit; Norton SA, São Paulo, SP, Brazil) in a water-cooled automatic polisher (APL4; Arotec, Cotia, SP, Brazil), to achieve their final diameter (10 mm) and thickness (0.5 mm), and cleaned ultrasonically with distilled water for 10 min and dried with compressed air. Disc thickness was measured with a 0.01mm accuracy digital caliper (Mitutoyo Corporation, Tokyo, Japan).

The LED curing unit output (Radii Cal; SDI, Bayswater, Victoria, VIC, Australia) was measured with a power meter (Ophir Optronics Inc., Danvers, MA, USA) at irradiance 1200 mW/cm².

Measurement of degree of conversion in resin cement

For analysis of degree of conversion, 10 cylindrical specimens were prepared by inserting Variolink Veneer light-curing resin cement (Ivoclar-Vivadent, shade HV+1) into a mold (1.0 mm deep / 7 mm diameter). Specimens were divided into two groups and cured for 20 s either through the CAD/CAM ceramic disc (n=5) or through the Press ceramic disc (n=5). Then they were washed in distilled water, dried with absorbent paper, placed in containers and stored at 37 °C for 24 hours.

The unpolymerized resin cement was accessed twice in order to set the bonding pattern of its monomeric state before sample analysis. Subsequently, all specimens were analyzed through Fourier Transform Infrared Spectroscopy – FTIR (Spectrum 100 Optica; Perkin Elmer, MA, USA), with attenuated total reflectance (ATR), using horizontal Zinc Selenite crystal (Pike Technologies, Madison, WI, USA), which acted as active substrate for the infrared rays.

Graphs were generated in the Spectrum software version 6.3.1 (PerkinElmer) for visualization and analysis. The FTIR spectrometer operated under the following conditions: 300-4000 cm⁻¹ wavelength, 4 cm⁻¹ resolution and 16 scans. Degree of conversion (DC) was calculated by comparing the aliphatic carbon-carbon double bond ratio (C=C) at 1640 cm⁻¹ and C=O at 1710 cm⁻¹ in cured and uncured materials, based on the following equation: DC (%) = (polymerized I - R / unpolymerized R) X 100; wherein R = peak at 1640 cm⁻¹/peak at 1710 cm⁻¹. These calculations were carried out in Microsoft Office Excel 2007 software (Microsoft Office Professional Edition 2007, Microsoft Corporation).

Measurement of microshear bond strength

The bars were separated into two groups - Press ceramic group (n=10) and CAD/CAM ceramic group (n=10) according to the manufacturing method. Bars were wet polished with 600- and 1200-grit silicon carbide abrasive papers (Norton SA, São Paulo, SP, Brazil) to produce a flat surface, dried, and air-abraded with 50 µm Al₂O₃ particles (Bioart, São Carlos, SP, Brazil). Then they were ultrasound cleaned in distilled water for 10 min and dried again. Subsequently, they were treated with 10% hydrofluoric acid for 20 s (Dentsply, Petrópolis, RJ, Brazil), subjected to RelyX Ceramic Primer (3M ESPE, St. Paul, MN, USA) application for 60 s and thoroughly air-dried. Adper Scotchbond Multi-Purpose Adhesive (3M ESPE) was applied with microbrush to all pretreated ceramic surfaces for 20 s, which were then thoroughly air thinned for 10 s and cured with LED curing light (Radii-Cal; SDI, Bayswater, VIC, Australia) at 1200 mW/cm² for 10 s through their respective ceramic disc, according to each processing technique. Two transparent cylindrical molds (Tygon tubing - TyG-03; Saint-Gobain Performance Plastic, Maime Lakes, FL, USA) (height 1 mm; internal diameter 0.75 mm) were positioned on the surface of each bar¹². A #5 exploratory probe (Hu-Friedy, Chicago, IL, USA) was used to apply the resin cement inside the molds. Each resin cement cylinder (two cylinders per bar) was photoactivated for 20 s through its respective ceramic disc and stored in distilled water at 37 °C. Twenty-four (24) hours later, the molds were removed with the aid of a scalpel blade to expose two cement cylinders with a bond area of 0.38 mm² (each). Bars were mounted on a steel device in a universal testing machine (DL2000; Emic, São José dos Pinhais, PR, Brazil). Load was applied at the base of the cylinder with the aid of a steel wire (0.2 mm diameter) at speed of 0.5 mm/min until fracture to determine the microshear bond strength. Each group contained 10 bars and each bar had two cylinders, providing a total of 20 cylinders per group. The average between the two cylinders on each bar was considered a specimen.

Statistical Analysis

Data were subjected to statistical analysis in the SAS System for Windows 9.0 (SAS Institute Inc., Cary, NC, USA). Measurement distribution was subjected to Kolmogorov-Smirnov test, after which

parametric tests were used. Degree of conversion (%) and microshear bond strength values (MPa) were subjected to Student's t-test, at 5% significance level.

RESULTS

Table 1 shows the mean values for degree of conversion and microshear bond strength. The mean of degree of conversion of resin cement was statistically higher when photoactivated through ceramic prepared with the CAD/CAM processing technique than through ceramic prepared with the Press processing technique. The microshear bond strength of resin cement was statistically higher when bonded to ceramic prepared with the CAD/CAM processing technique than ceramic prepared with the Press processing technique.

Table 1 – Mean and standard deviation (in parentheses) of degree of conversion (%) and microshear bond strength (MPa) of resin cement.

Ceramic Group (Processing Technique)	Degree of Conversion	Microshear Bond Strength
CAD/CAM	44.74 (2.96) A	22.18 (1.01) A
Press	25.71 (1.61) B	19.83 (0.42) B

Different letters indicate statistically significant differences between mean values (Student's t-test; $p < 0.001$).

DISCUSSION

Lithium disilicate-based ceramics have been extensively used to manufacture full and partial monolithic restorations¹³. Two processing techniques are available: pressing and CAD/CAM technology¹⁴⁻¹⁶. Pressing technology is a classic methodology used to manufacture duplicate restorations in wax, which is inserted in a special investment cast. Next, flame-volatilized ceramic ingots are injected into the cast under high vacuum so that the shape of the final restoration is identical to that of the wax piece (lost-wax technique)¹⁵. CAD/CAM systems use digital workflow to achieve final restorations through digital casts. Restoration and the milling process are designed in computer software, which provides high precision, efficiency and accuracy, in addition to reducing processing time¹⁷. Thus, the current study assessed the degree of conversion of resin cement when photoactivated through ceramic prepared using two different processing techniques (Pressing and CAD/CAM)

and the microshear bond strength of resin cement to ceramics prepared in these two ways.

It is known that resin cement polymerization can be affected if light does not reach it¹⁸. Thickness and shade are two of the factors capable of hindering polymerization processes^{19,20}. Only discs with similar ceramic thickness (0.5mm) and shade (HV+1) were used in the current study to prevent these factors from affecting the degree of conversion of the resin cement. The photopolymerizing device was placed in contact with the ceramic bars in order to avoid any differences in the distance between the ceramic type and light. The same light-curing unit (LED with 1200 mW/cm²) and exposure time (20 s) were used for the resin cement. However, irradiance after the interposition of ceramic discs was attenuated to 1110 mW/cm² in the CAD/CAM processing technique and to 1020 mW/cm² in the Press processing technique. Few studies available in the literature have shown similar outcome for material microstructure. According to Alkadi and Ruse²¹, materials subjected to the pressing technique had increased crystallization rates, whereas Peng et al.²² found larger grain size of lithium-disilicate crystals in the pressable form (CAD/CAM — 0.1–1.0mm <Pressed — 3–6mm). Both complete crystallization and grain size of lithium-disilicate crystals can potentially affect the mechanical performance of the material. Based on the current results, ceramic manufacturing technique (Press or CAD/CAM) significantly influenced the degree of conversion of the resin cement, which was statistically higher for resin cement photoactivated through ceramic processed with the CAD/CAM technique than for resin cement photoactivated through ceramic processed with the press technique.

Bond strength between resin cements and lithium disilicate ceramics is an important factor for ensuring long-lasting dental restorations⁶. Stress transfer to the remnant tooth through the ceramic restoration depends on adequate bonding interface, which can prevent failures²³. The present study has found differences in the bond strength of resin cement to the lithium disilicate ceramics manufactured with different techniques. Findings in the present study are clinically acceptable, regardless of the material resistance to microshear values (13 MPa–30 MPa)^{24,25}. Previous studies have found that material resistance declines over time^{24,25}. However, the present study did not use simulated aging to assess

microshear bond strength, which could be seen as a research limitation. Thus, further studies are needed focused on assessing bond strength after simulated aging based on different processing techniques. Based on results in the current study, the null hypotheses were rejected because different degrees of conversion [1] and microshear bond strengths [2] were found for the two processing techniques.

CONCLUSIONS

Based on the methodology and materials used, and

on results observed in the current study, we conclude that:

1. The degree of conversion of resin cement was higher when it was photoactivated through ceramic prepared with the CAD/CAM technique than through ceramic prepared with the press technique.
2. The microshear bond strength of resin cement was greater when bonded to ceramic prepared with the CAD/CAM technique than ceramic prepared with the press technique.

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Enzyme production by *Candida albicans* and *Candida dubliniensis* in periodontal HIV-positive patients receiving and not receiving antiretroviral therapy

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ABSTRACT

Candida dubliniensis (Cd) and *Candida albicans* (Ca) are the most frequently isolated yeasts in HIV+ patients. Some of the enzymes produced by these yeasts are considered virulence factors since they contribute to pathogenicity of *Candida* spp.

The aim of the present study was to compare production of enzymes such as phospholipase (Ph), proteinase (P), and hemolysin (H) by Cd and Ca strains isolated from periodontal HIV-positive patients receiving and not receiving highly active antiretroviral therapy (HAART).

Subgingival biofilm samples were obtained using paper points, and a sample of oral mucosa was taken using a swab.

Phenotypic and molecular methods were used to isolate 39 strains of *Candida*, including 25 strains of Cd and 14 strains of Ca, obtained from 33 periodontal pocket samples and 6 oral mucosa samples collected from 15 HIV+ patients (8 receiving and 7 not receiving HAART). Malt egg-yolk agar, albumin agar and blood agar were used to evaluate pH, P and H production respectively. The strains were inoculated in duplicate

and incubated at 37 °C. Colony and halo diameters were measured.

A greater proportion of Ca was observed in patients not receiving HAART, and a higher proportion of Cd was observed in those under HAART, $\chi^2 p < 0.001$. Phospholipase production was observed in 92.9% percent of isolated Ca strains but in none of the isolated Cd strains. Proteinase production was high in Ca and Cd strains isolated from patients not receiving HAART. Hemolysin production was observed in all the studied strains, though it was significantly higher ($p=0.04$) in Ca and Cd strains isolated from patients not receiving HAART.

To sum up, the proportion of *Candida dubliniensis* strains was highest in the subgingival biofilm of patients receiving HAART, and Cd strains were found to express fewer virulence factors than Ca strains.

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Keywords: *candida albicans*- HIV- periodontitis- antiretroviral therapy.

Producción enzimática de *Candida albicans* y *Candida dubliniensis* de pacientes con periodontitis VIH+ con y sin tratamiento antirretroviral

RESUMEN

Las levaduras más aisladas en pacientes VIH+ son *Candida dubliniensis* (Cd) y *Candida albicans* (Ca). Algunas de sus enzimas constituyen factores de virulencia ya que favorecen la diseminación tisular.

El objetivo fue comparar la producción de enzimas como fosfolipasa (F), proteinasa (P) y hemolisina (H) en cepas de Cd y Ca aisladas de pacientes VIH+ tratados y no tratados con antirretrovirales (TARGA).

Se realizó la toma del biofilm de placa subgingival con conos de papel y la muestra de la mucosa bucal con hisopo.

Se aislaron y tipificaron por métodos fenotípicos y moleculares 39 cepas: 25 de Cd y 14 Ca, obtenidas 33 de bolsos periodontales y 6 de mucosa bucal de 15 pacientes VIH+ (8 con y 7 sin tratamiento). Se utilizó agar malta con yema de huevo, agar albúmina y agar sangre para demostrar la producción de F, P y H, respectivamente. Se inocularon por duplicado e incubaron a 37°C. Se midieron los diámetros de las colonias y los de hidrólisis alrededor de las mismas.

Se observó mayor proporción de Ca en los pacientes sin tratamiento y mayor proporción de Cd en los con tratamiento; $\chi^2 p < 0.001$.

El 92,9% de las Ca estudiadas, fueron productoras de fosfolipasa. En tanto que ninguna Cd produjo la enzima.

En cuanto a la producción de proteinasa se observa una alta producción tanto en las cepas de Ca, como en las Cd aisladas en los pacientes no tratados.

Todas las cepas estudiadas produjeron hemolisina, observándose una diferencia estadísticamente significativa ($p=0,04$) en ambas especies a favor de la alta producción de

la enzima en las cepas obtenidas de pacientes no tratados. Podemos concluir que en el biofilm subgingival, en los pacientes bajo TARGA, se aíslan mayor proporción de Candida dubliniensis las cuales expresan menos factores de virulencia.

Palabras clave: *candida albicans- VIH- periodontitis- terapia antiretroviral.*

INTRODUCTION

Colonization by *Candida* species begins within the first few days of life. Although the oral mucosa is a reservoir, *Candida* spp. are also present in the biofilm of periodontal pockets of patients with periodontitis, endodontic infection and periimplantitis¹.

In 30 to 50% of subjects, *Candida* spp. colonize the oral cavity and the digestive tract, and are more frequent in lactating infants, elderly subjects who use a dental prosthesis, people who have received antibiotic treatment or chemotherapy, patients with poorly controlled diabetes, and hospitalized and immunosuppressed patients².

Asymptomatic colonization of the oral mucosa is more prevalent among HIV+ subjects and considered an important predisposing factor for candidiasis.

The advent of highly active antiretroviral treatment (HAART) has markedly improved the immune system of HIV+ patients, substantially decreasing the incidence of opportunistic infections³.

The most frequently isolated yeasts from the oral cavity of HIV+ patients are *Candida albicans* and *Candida dubliniensis*. These species share a number of phenotypic features, such as the formation of green colonies in chromogenic media and the ability to produce chlamydospores and pseudofilaments^{4,7}. The virulence factors of these yeasts include phenotypic change, dimorphism, adherence capacity and secretion of lytic enzymes such as phospholipase, proteinase and hemolysin, among others^{8,9}.

Production of these enzymes can therefore be an important determining factor of the potential of these microorganisms to produce invasive infection in certain groups of individuals, as is the case of immunocompromised patients¹⁰.

The aim of the present study was to describe and compare enzyme production by commensal strains of *Candida albicans* and *Candida dubliniensis* obtained from different oral ecological niches in periodontal HIV+ patients, receiving and not

receiving highly active antiretroviral therapy (HAART).

MATERIALS AND METHODS

The study population comprised adults living with HIV/AIDS residents in the Buenos Aires City, who attend dental care services. The sample included 15 HIV patients (8 receiving and 7 not receiving HAART) seen at the High-Risk Patients Oral Care Unit (CLAPAR I), School of Dentistry, University of Buenos Aires, who underwent oral examination after they had voluntarily signed an informed consent form. The study was approved by the Ethics Committee of the School of Dentistry, University of Buenos Aires (Resolution 21/11/2012-33).

Patients aged 18 to 60 years, diagnosed with HIV infection (confirmed by 2 positive ELISA tests and 1 Western Blot or similar), with a minimum 6 teeth and one site per quadrant with a probing depth equal to or higher than 5mm, attachment loss, and positive bleeding on probing, were included in the study. Only HAART patients who had remained on the same regimen the whole year prior to study enrolment were included.

Patients with systemic disease unrelated to HIV infection prior to or presenting during the study, receiving antibiotic/antifungal and/or oral-antiseptic treatment within 3 months prior to microbiological testing, showing mucosal candidiasis lesions, receiving oral care at other private or public oral health care services, and/or who smoked were excluded from the study.

Under adequate lighting conditions and using standardized instruments (a Marquis color-coded probe and a dental mirror), a calibrated researcher assessed the following clinical parameters of periodontal disease at 6 different sites per tooth (3 on the buccal aspect and 3 on the palatal or lingual aspect): presence of biofilm, probing depth (PD), clinical attachment level (CAL) and bleeding on probing (BP).

The patients were then instructed to rinse their mouth with distilled water. The site showing the worst periodontal parameters in each quadrant was identified. Following removal of the supragingival plaque using a curette and tooth isolation with cotton rolls, subgingival biofilm samples were collected using 4 paper points placed one at a time into the depth of the pocket for 20 seconds. The obtained samples were placed in reduced transport fluid (RTF) medium. A sample of oral mucosa was obtained using a swab, which was placed in a tube containing RTF medium.

All the samples were submitted to the microbiology laboratory for processing.

Microbiological Procedures

The samples were homogenized by shaking in a vortex mixer; 50 µl of each suspension were seeded in CHROMagar Candida® for presumptive identification of the species⁷.

C. albicans and *C. dubliniensis* were distinguished by using conventional phenotypic methods^{5,6}: cultures on milk-tween 80 agar for detection of germ-tubes, pseudofilaments, and chlamydoconidia¹¹, cultures on Sabouraud Dextrose Agar (SDA) incubated at 45 °C¹², and cultures on Staib agar for production of chlamydoconidia¹³.

Molecular identification

Genomic DNA was extracted from pure and fresh 24-h cultures in SDA. Cell lysis was reached by heating at 100 °C and centrifuging at 16000g.

Two species-specific primers derived from the internally transcribed spacer (ITS) region (comprising the ITS1, 5.8s rRNA and ITS2 regions) as described by Asadzadeh et al.¹⁴ were used.

Amplification reactions were performed by real time PCR (qPCR) using 2X SYBR Green Supermix in a 10 µl final volume, 10 µM of each primer, and 2 µl of genomic DNA, in a thermal cycler (CFX96 C1000 Touch, BioRad, Hercules, CA, USA). PCR cycling conditions were as follows: denaturation at 95 °C for 5 minutes, followed by 39 amplification cycles at 95 °C for 15 seconds, 60 °C for 30 seconds, 65 °C for 5 minutes, and 95 °C for 5 minutes.

The amplification process was evaluated using MCA (melting curve analysis) according to Asadzadeh et al.¹⁴.

Detection of enzyme activity

All the isolated strains were seeded in SDA and incubated at 37 °C for 18h. The cultures were used to prepare suspensions adjusted to 1 McFarland standard (1 x 10⁶ CFU/ml).

Phospholipase, proteinase and hemolysin production was detected using malt agar supplemented with sterile egg yolk emulsion (2%)⁸, bovine serum albumin agar⁸, and SDA with sterile sheep blood (7%)¹⁵, respectively.

Sterile paper discs were placed on the surface of each dish containing the corresponding medium, inoculated with 10 µl of the suspension, and incubated under aerobic conditions at 37 °C. The assays were performed in duplicate.

Phospholipase production was assessed at 72 h and was considered positive when a zone of precipitation was observed around the colony.

The precipitation zone (Pz) value was calculated as the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone¹⁶. Enzyme production was classified according to the Pz value as follows: Pz value = 0.35 – 0.5 (high producers), Pz value = 0.51 – 0.74 (moderate producers); Pz value = 0.75 – 0.9 (low producers); and Pz value = 1 (negative)¹⁷.

Hemolytic halos were measured, and the enzymatic activity Pz value was calculated according to the ranges defined above.

Proteinase production was determined seven days post-incubation; the dishes were stained with a 0.6% amido black solution and destained with acetic acid⁸. The presence of a hydrolysis zone around the colony showed positive proteinase activity.

Candida albicans ATCC 10231 was used as positive control for the production of all three enzymes, *Candida glabrata* ATCC 90030 was used as negative control for phospholipase, and *Candida parapsilopsis* ATCC 22019 was used as negative control for hemolysin and proteinase.

Statistical Analysis

Categorical variables were analyzed as frequencies and percentages using 95% confidence intervals, and comparisons were established using Chi square test with Yates' continuity correction. In all cases, statistical tests for independent samples were used and level of statistical significance was less than 5% to reject the null hypothesis.

All statistical analyses were performed using SPSS software version 26, MS Excel 2016, Epidat 4.0.

RESULTS

As shown by the clinical records, 7 (46.7%) of the 15 studied patients were women and 8 (53.3%) were men, and all 15 patients contracted the virus by sexual transmission.

Mean age of the patients was 41 years ($SD \pm 10$ years) and age range was 28 to 58 years. Analysis of the population according to age group showed that 20.0% were below the age of 30 years, 33.0% were between the age of 30 and 40 years, and 47.0% were over the age of 40 years.

Forty-seven percent of patients had been diagnosed with HIV within 10 years prior to the study, and 53.0% had been diagnosed more than 10 years before enrolment in the study. Eight patients (53.0%) were receiving HAART and 7 (47.0%) were not receiving HAART at the time of the study; 50% of patients receiving HAART had been under treatment for more than 10 years.

A total 75 samples, including 60 samples (80.0%) of subgingival biofilm and 15 samples (20.0%) of oral mucosa, were obtained from 15 patients. A total 39 strains of *Candida* genus were isolated: 14 (35.9%) *Candida albicans* and 25 (64.1%) *Candida dubliniensis* strains. Distribution according to species and localization is shown in Table 1.

Table 1. Frequency and percentage distribution of *Candida* spp. according to localization.

Samples	<i>Candida</i> spp. isolate		
	<i>Candida albicans</i>	<i>Candida dubliniensis</i>	Total strains
Periodontal Sites	12 (85.7%)	21 (84.0%)	33 (84.6%)
Oral Mucosa	2 (14.3%)	4 (16.0%)	6 (15.4%)
Total	14 (35.9%)	25 (64.1%)	39 (100.0%)

Both species were isolated from the same periodontal site in two patients, one receiving and one not receiving HAART.

No statistically significant differences in the distribution of *Candida* species or localization were observed; chi square test $p=0.887$.

Analysis of the isolated species according to presence or absence of treatment showed 3 *C. albicans* (13%) and 20 *C. dubliniensis* (87%) strains in the 8 patients receiving HAART, and 11 *C. albicans* (68.8%) and 5 *C. dubliniensis* (31.2%) strains in the 7 patients not receiving HAART (Fig 1).

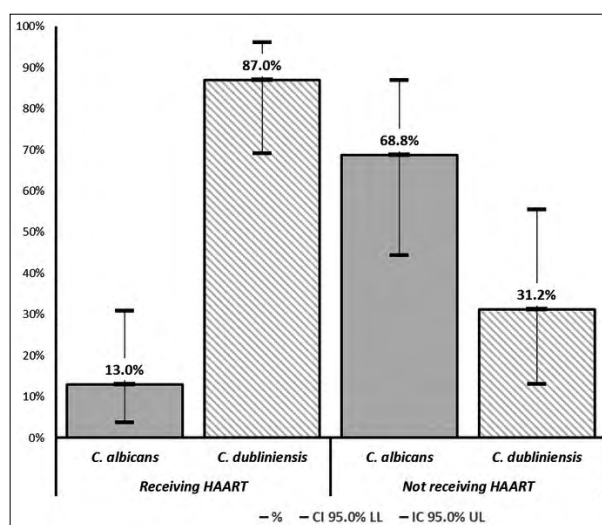


Fig. 1: Percentage distribution of *Candida* spp. according to presence/absence of HAART.

A greater proportion of *Candida albicans* was observed in patients not receiving HAART, and a higher proportion of *Candida dubliniensis* was observed in patients under HAART; Chi square test $p < 0.001$.

In view of the distribution of phospholipase and proteinase production, the statistical analysis was performed considering positive or negative production (presence /absence) and Pz values were not considered.

Phospholipase production was observed in 13 (92.9%) of the 14 *Candida albicans* strains, 11 of which were isolated from patients not receiving HAART and 2 from patients under HAART (Table 2). However, no phospholipase production was observed in any of the *Candida dubliniensis* strains from either group.

Table 2. Frequency and percentage distribution of phospholipase production by *Candida albicans* strains isolated from patients receiving and not receiving HAART.

Group	Phospholipase production	
	Positive	Negative
Receiving HAART	2 (66.7%)	1 (33.3%)
Not receiving HAART	11 (100.0%)	0 (0.0%)
Total	13 (92.9%)	1 (7.1%)

Proteinase production was higher in both *Candida albicans* and *Candida dubliniensis* strains isolated from patients not receiving HAART; Chi square test, $p < 0.001$ (Fig. 2 and Table 3).

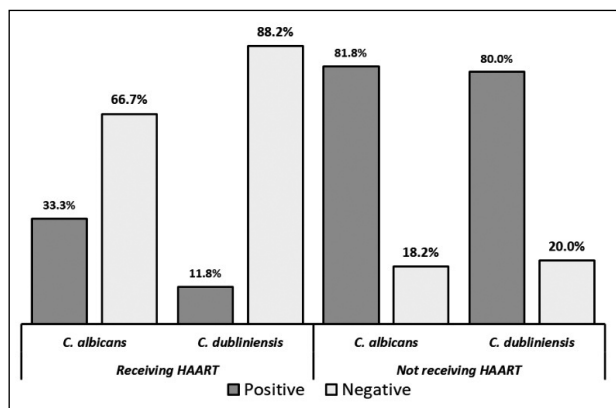


Fig. 2: Percentage distribution of proteinase production by *Candida albicans* and *Candida dubliniensis* strains isolated from patients receiving and not receiving HAART.

Table 3. Frequency and percentage distribution of proteinase production by *Candida spp.* isolated from patients receiving and not receiving HAART.

Group	Proteinase production	
	Positive	Negative
Receiving HAART	3 (15.0%)	17 (85.0%)
Not receiving HAART	13 (81.2%)	3 (18.8%)
Total	16 (44.4%)	20 (55.6%)

As regards hemolysin production, all the studied strains were found to produce the enzyme, and production was significantly higher ($p=0.04$) both in *Ca* and in *Cd* strains isolated from patients not receiving HAART (Fig. 3 and Table 4).

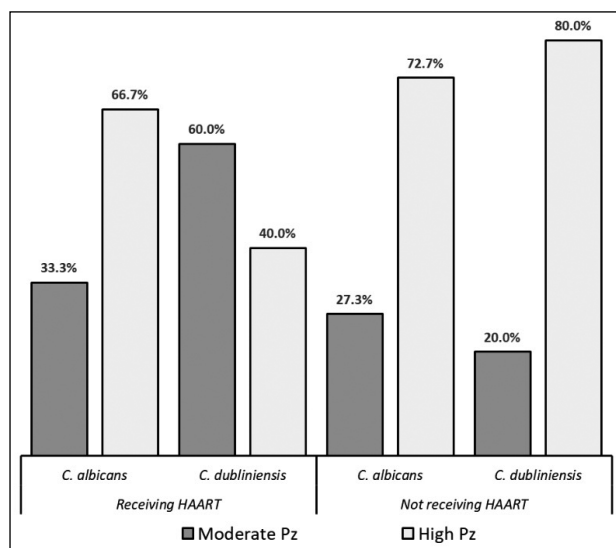


Fig. 3: Percentage distribution of hemolysin production by *Candida albicans* and *Candida dubliniensis* strains isolated from patients receiving and not receiving HAART.

Table 4. Frequency and percentage distribution of hemolysin production by *Candida spp.* isolated from patients receiving and not receiving HAART

Group	Hemolysin production	
	Moderate Pz	High Pz
Receiving HAART	13 (56.5%)	10 (43.5%)
Not receiving HAART	4 (25.0%)	12 (75.0%)
Total	13 (92.9%)	1 (7.1%)

DISCUSSION

The distribution of age, sex, and mode of acquisition of HIV infection was similar in both study groups of patients. Of note, all the studied yeast strains were isolated from oral mucosa and periodontal pockets of patients with no clinical manifestation of candidiasis.

In the present study, a larger number of strains of both *Candida albicans* and *Candida dubliniensis* was isolated from subgingival biofilm (Table 1).

Although the role of yeasts in the pathogenesis of periodontitis has not been fully elucidated to date, a number of authors have reported the presence of yeasts in periodontal pockets of subjects with chronic periodontitis, including both immunocompetent and immunocompromised patients^{18,19}.

There are reports in the literature of *Candida dubliniensis* isolates from non-HIV patients²⁰⁻²².

Ursua B. *et al.* found that *C. albicans* and *C. dubliniensis* were capable of colonizing periodontal pockets, regardless of pocket depth, in non-HIV patients with chronic periodontitis²³.

According to Vieira Colombo AP *et al.*, subgingival biofilm can be a source of development and dissemination of systemic infections²⁴. Periodontal scaling could cause fungemia, which in turn could lead to serious complications, depending on the immunological status of the patient.

In the present study, a larger number of *Candida dubliniensis* strains were isolated from HIV+ patients receiving HAART (Fig. 1), suggesting that compliance with treatment would result in homeostasis between the immunological status of the patient and the yeasts, decreasing the development of opportunistic infections, particularly candidiasis. Vilela MM *et al.* showed that *Candida dubliniensis* is less virulent than *Candida albicans*²⁵.

Paula SB *et al.* recovered fewer *Candida dubliniensis* isolates from HIV+ patients receiving HAART²⁶.

The detection of *Candida* species in periodontal pockets may be due to the ability of these yeasts to adapt to changes in the environment by altering pH, oxygen concentration, and nutrient availability²⁷. This enables them to coexist in the subgingival biofilm and coaggregate with periodontopathic bacteria, as shown in a paper recently published by our research team²⁸.

According to Lourenço *et al.*, the discrepancies among studies regarding the prevalence of *Candida* spp. could be due to the method used to collect the study samples, such as swabbing, collecting total saliva or oral rinses.²⁹ In line with Samaranayake *et al.*, the authors found that the oral rinse technique was the most sensitive for detecting *Candida* spp.³⁰ We consider that collecting subgingival biofilm samples is an adequate method to recover *Candida* spp. Jewtuchowicz *et al.* isolated *Candida* spp. from subgingival samples obtained using a curette from immunocompromised patients²².

Several authors have reported coinfection by *Candida dubliniensis* and other *Candida* species, particularly *Candida albicans*.³¹ In the present study, we observed coinfection of a single site by both species in two cases: one patient under HAART and the other patient not receiving HAART.

All the strains were identified using phenotypic methods, and then confirmed using molecular biology techniques. Pineda G. *et al.* suggested that the combined use of at least three phenotypic differentiation techniques might be a valid alternative for laboratories that do not have access to molecular diagnostic methods⁶.

The transition of yeasts from commensal to opportunistic pathogens is the result of a number of mechanisms to overcome the host's defense barriers, including adhesion, biofilm formation and production of hydrolytic extracellular enzymes such as phospholipase and proteinase; all these properties are considered virulence determinants.

Aspartic proteases play an important role in the degradation of the mucosal (collagen, keratin,

and mucin) as well as the immune components (cytokines, antibodies, and complement), facilitating invasion of the host's tissues⁸.

Phospholipase can damage the host's cell membrane by degrading its constitutive lipids. Phospholipase production can therefore be an important factor in determining the ability of *Candida* spp. to invade tissues¹⁰.

Hemolysin production was observed in all the strains isolated in the present study. Manns *et al.* showed that iron is found intracellularly as ferritin or contained in the heme group, and that small amounts of extracellular iron are found bound to transport proteins such as transferrin and lactoferrin. Microorganisms also require iron to grow, and their ability to acquire iron is essential to their survival and to establishment of infection⁹. We understand that hemolysin production is a survival rather than a virulence factor.

The results of the present study showed that both species isolated from patients not receiving HAART produced proteinase (Fig. 2), whereas strains recovered from patients under HAART were low proteinase producers. As suggested by Thomas *et al.*³² and Ribeiro *et al.*³³, this difference could be explained by the use of protease inhibitors in HAART. Nevertheless, other authors posit that the presence of protease inhibitors in HAART does not affect the production of this enzyme³⁴⁻³⁶.

In the present study, phospholipase production was observed in all the *Candida albicans* strains isolated from patients not receiving HAART, but in none of the *Candida dubliniensis* strains from either group of patients. Our findings are in line with studies testing different quantities of *Candida dubliniensis* strains^{20,3,21}.

CONCLUSIONS

A larger proportion of *Candida dubliniensis* strains were isolated from the subgingival biofilm of patients receiving HAART, and these strains had fewer virulence factors.

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Prospective clinical study of transverse development with orthodontics with self-ligating brackets

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ABSTRACT

Orthodontics with low friction, low force, passive self-ligating brackets produces alveolar-dental remodeling, resulting in an increase in the transverse diameter of the dental arches, especially in the premolar sector.

The aim of this study was to compare the modifications of the transverse diameter in the pre- and post-dental alignment cast models with orthodontics with passive self-ligating brackets in patients with moderate to severe dental discrepancy.

The study included 28 patients of both sexes aged 16 to 48 years with dental discrepancies between -6 and -16 mm, treated with self-ligating Damon brackets and thermally activated Nickel-Titanium-Copper arches.

With a digital pachymeter, Mitutoyo brand, five measurements were taken per dental arch: distance between canines (C), first premolars (1PM), second premolars (2 PM), first molars (1M)

and second molars (2M), before and after orthodontic alignment. The variations were statistically evaluated by Student T Test for paired samples.

Average distance between teeth varied with dental alignment in both jaws. The greatest increases in transverse diameter were recorded in the premolar areas. Minor though statistically significant variations also occurred in the 2M of the maxilla and in the C of the mandible. In alignment with passive self-ligating brackets, there is an increase in the transverse diameter due to the development of the dental arches, mainly in the premolar sector of both jaws and also at the level of the canines in the mandible.

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Keywords: orthodontic brackets- malocclusion- tooth movement techniques.

Estudio clínico prospectivo del desarrollo transversal con ortodoncia con brackets autoligables

RESUMEN

La ortodoncia con brackets autoligables pasivos, de baja fricción y baja fuerza, produce remodelación alvéolo-dentaria; con aumento del diámetro transversal de las arcadas, especialmente en el sector de los premolares. El objetivo de este trabajo fue comparar las modificaciones del diámetro transversal en los modelos de yeso pre y post alineación dentaria con ortodoncia con brackets autoligables pasivos en pacientes con discrepancia dentaria moderada a severa. El estudio incluyó 28 pacientes de ambos sexos de entre 16 y 48 años de edad con discrepancia dentaria entre -6 y -16mm, tratados con brackets autoligables sistema Damon y arcos termo activables de Níquel-Titanio-Cobre. Con un paquímetro digital marca Mitutoyo, se tomaron 5 medidas por arcada: distancia entre caninos (C), primeros y segundos premolares (1 PM y 2 PM) y primeros y segundos molares (1M y 2M); pre

y post alineación ortodóncica. Las variaciones registradas fueron evaluadas estadísticamente mediante Student T Test para muestras apareadas. La distancia promedio entre dientes varió con la alineación dentaria en ambos maxilares. Los mayores aumentos del diámetro transversal se registraron en las zonas de los premolares. Se produjeron también variaciones menores, aunque estadísticamente significativas, en los 2M del maxilar superior y en los C del maxilar inferior. En la alineación con brackets autoligables pasivos, hay incremento del diámetro transversal por desarrollo de las arcadas, principalmente en el sector premolar de ambos maxilares y en el maxilar inferior también a nivel de los caninos.

Palabras clave: brackets de ortodoncia- maloclusión- técnicas de movimiento dentario.

INTRODUCTION

Since self-ligating brackets appeared in 1935, numerous papers have been published with the intention of determining the advantages of the self-ligating system over the conventional¹⁻⁶.

Self-ligating brackets reduce ligating friction, and the use of super elastic archwires enables the application of light forces that produce tooth movements. These are the forces of choice in orthodontic treatments. The clinical response of low friction systems and light forces with self-ligating brackets is different from that obtained in friction systems with conventional brackets. In a comparative study between treatment with conventional and self-ligating brackets, Herradine⁵ found lower incisor inclination in self-ligating braces and expansion in both systems.

Reddy et al.⁷ found that for space closing, treatments with self-ligating brackets were more efficient than conventional brackets, and the alignment occurred with less incisor inclination.

Lineberger⁸ carried out a study of digital models in which a control group of 25 untreated patients was compared to 25 patients treated with self-ligating brackets (DAMON). In the Damon-treated group, there was an increase in the transverse diameter in the premolar area from 2 to 2.2 mm. There is no reference in the inclusion criteria to the need for space required for alignment (crowding), patients with Angle Class I canine and molar were included. Morais et al.⁹, found a significant increase in the transverse diameter in the dental arches in patients that were treated with self-ligating brackets, especially between first premolars. It determined that the widening occurred mainly by buccal inclination. Our hypothesis is that in patients with moderate to severe crowding treated with self-ligating brackets, the greatest gain in space occurs in the premolar area, achieving the natural dental arch shape, giving space to the aligning teeth.

The aim of this study is to evaluate in pre- and post-alignment models the differences in the distance in both jaws between canines, first premolars, second premolars, first molars and second molars.

MATERIALS AND METHODS

The project was approved by the Bioethics Commission of the School of Dentistry of the University of Buenos Aires, code CO22, 2012-2014.

The study began with 31 patients aged 16 to 48 years old who were treated at the Orthodontic Department of the School of Dentistry of the University of Buenos Aires. Patients with systemic diseases, joint disorders, periodontitis, untreated caries, mixed or temporary dentition and with impacted or absent teeth (except third molars) were excluded. All patients signed their informed consent to participate in this project. Three patients discontinued treatment for personal reasons, so the final number of subjects included in the study was 28 – 10 male and 18 female. These 28 patients needed dental arch expansion due to moderate to severe dental crowding (dental discrepancy between -6 and -16 mm).

Patients were treated with the Damon System (low friction system), Damon II brackets (A-Company) and copper nickel-titanium archwires (Damon Cu-NiTi 0.014 and 0.016), Damon format archwires. Each treatment plan was prepared according to the initial diagnosis with or without indication of dental extractions. If necessary, the extractions were performed after the alignment stage. Routine diagnostic studies were performed (Panoramic x-ray, lateral cephalometric radiograph; cephalometric tracings, clinical examination and dental models). The dental arch was considered aligned when it was possible to place the rectangular arch. No tooth extractions or interproximal stripping were performed in this initial stage of treatment. Impressions were taken for plaster casts for evaluation of the post-alignment stage.

The distance between homologous teeth was measured on the casts of the maxilla and mandible before and after treatment (Fig1). The measurement method was described in a previous work.¹⁰

A Mitutoyo Digimatic NTD12-6" C brand digital caliper was used; Mitutoyo Corp. Tokyo, Japan. Three calibrated operators performed the measurements.

The differences between pre- and post-treatment were statistically evaluated using the two-tailed Paired-Samples Student's T-Test.

RESULTS

The measurements are shown in Table 1. The differences between the pre- and post-treatment measurements in the maxilla were statistically significant for 2PM, 1M and 2M. In the mandible, significant differences were found for canines and premolars.

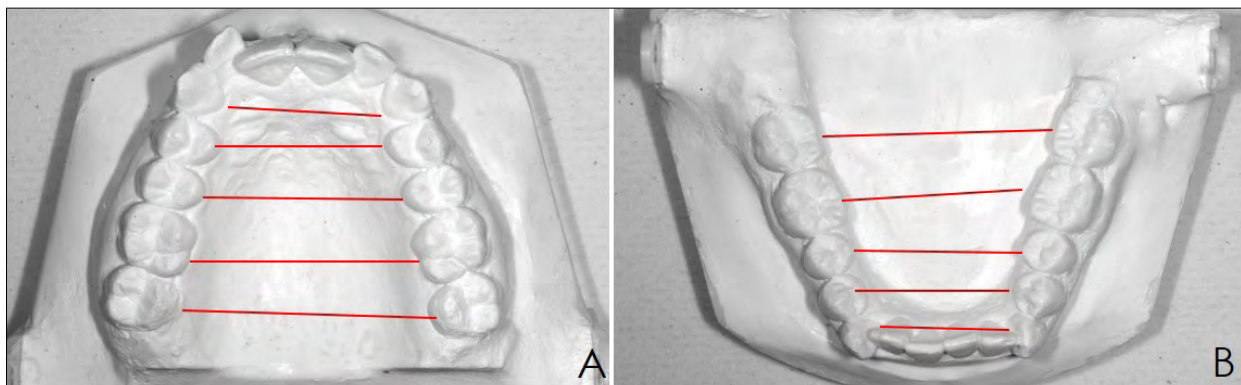


Fig. 1: Five measurements were taken per patient per jaw, between canines, first premolars, second premolars, first molars, and second molars, before and after dental alignment. A: Maxilla B: Mandible.

DISCUSSION

The treatment with the Damon System uses self-ligating brackets and Damon Copper Ni-Ti archwires¹ (copper-nickel-titanium) with thermal-reactive properties, which deliver very low force that could be controlled by the musculature. The size of the arches is the same for both jaws, being larger in relation to other formats such as the "True Arch" of the Roth philosophy¹¹ or the formats established for treatment with the MBT System designed to respect the original shape of each arch: ovoid, narrow or square, with different sizes for maxilla and mandible. The material used in the alignment phase is nickel titanium.¹²

Clinical studies comparing self-ligating and conventional brackets include patients with mild to moderate crowding because a patient with severe crowding could not be treated with conventional orthodontics without having to be initially extracted.^{6,13-19}

These studies do not evaluate the results of treatment with self-ligating brackets in patients with crowding greater than 7 mm and the need for transverse arch development.

In the present study, moderate to severe crowding was used as an inclusion criterion to evaluate the results of treatment with self-ligating brackets by comparing dental arches before and after alignment with orthodontics.

Inter-canine distance had minimal variation for the maxilla, with a large deviation between cases. In the mandible, the pre- and post-treatment difference increased slightly on average, with less deviation between cases.

Regarding the variations in the premolar sector, a significant increase in transverse diameter was found for both premolars and in both dental arches.

Vajaria et al.¹⁴ included the evaluation of the distance between premolars, which was not taken into account in previous studies. In a retrospective study, they compared patients treated with the conventional and self-ligating systems. Despite the fact that the population studied was different from that of the present study, both in the inclusion criteria and in the lack of description on the degree of crowding, their results have the same trend as ours. It is inferred that transverse development occurs according to the need for space presented by each patient according to the compression or crowding in their dental arches. Due to the action exerted by the deflection of the archwire, remodeling occurs in the dental arches according to the space required by the teeth. The greatest transverse gain is found in the inter-premolar distance, which will be greater in the patient with the highest compression. The greatest compression in the premolar sector would be in relation to the lack of natural development of the dental arches,

Table 1. Variation of the distances between contralateral teeth at the end of alignment phase (N=28)

	Maxilla		Mandible	
	X± SD*	p value**	X± SD*	p value**
Canines	0.29±2.44	0.34	0.82±1.28	<0.01***
1st premolars	3.14±1.24	<0.01***	1.97±1.93	<0.01***
2nd premolars	2.55±1.73	<0.01***	2.1±1.59	<0.01***
1st molars	0.31±1.24	0.19	0.38±1.46	0.23
2nd molars	-0.72±1.49	<0.01***	0.07±2.2	0.86

*Mean ± standard deviation. ** T Student Test, paired data.

***statistically significant difference

associated with alteration of oral functions. In the maxilla, distance increased slightly between first molars and decreased between second molars, with a statistically significant difference between the values. This would be related to the transverse compression observed in the pre-treatment models between premolars and first molars. The second molar presents a position toward the vestibular in the dental arch at the transverse level, possibly because it erupts protected from abnormal muscular activity by anatomical landmarks. In the mandible, both inter-molar distances increased slightly, with no statistically significant difference. The greatest modification in the transverse diameter of both upper and lower dental arches occurs at the level of the inter-premolar distance, coinciding with the studies by Cattaneo,¹⁰ Lineberger⁸ and two previous

studies published by our group⁹⁻¹³. The originality of the present study is the inclusion in the sample of patients with moderate to severe crowding, in whom the teeth could remain in their basal areas without extractions, a result that could not have been achieved without the transverse development of the dental arches.

CONCLUSION

In alignment with passive self-ligating brackets in patients with moderate to severe crowding, there is a significant increase in transverse diameter due to the development of the dental arches, mainly in the premolar sector in both jaws. The greater transverse development in the maxilla would indicate that the appliance enlarges the dental arch that most needs it, even if the same arch format is used.

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Post preparation: cleanness achieved by different irrigating protocols

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ABSTRACT

The aim of this study was to evaluate the ability of different irrigation protocols to remove from the walls of the root canal and entrance to dentinal tubules the smear layer produced during preparation for a fiber post. Fifty decoronated human lower premolars were treated endodontically and the apical third of the canal was filled with a single gutta-percha point using warm vertical compaction. Ten millimeters were left free for post preparation, which was done by sequential use of a Largo #1 drill and Exacto #3 pilot drill (Angelus, Brazil), with irrigation with distilled water upon each instrument change. Samples were distributed randomly into 5 groups (n=10). G 1: No irrigation after post preparation. G 2: Distilled water activated for 60 s + distilled water. G 3: 5.25% NaOCl 15 s + distilled water. G 4: 17% EDTA (Farmadental, Argentina) 60 s + 5.25% NaOCl 15 s + distilled water. G 5: 10% polyacrylic acid (Densell) 15 s + distilled water. Irrigant was activated mechanically with a

low-speed conical brush. Roots were then split longitudinally in vestibular-lingual direction. Each surface was observed under SEM in its different thirds. Microphotographs were taken at 150X and 600X magnification and results analyzed statistically using Kruskal Wallis and Friedman tests ($p < 0.05$). The results [mean (standard deviation)] were: for 150X: **G1** 11.00 (1.33), **G2** 7.50 (3.13), **G3** 6.30 (2.58), **G4** 2.20 (2.80), **G5** 4.30 (1.50), and for 600X **G1** 11.40 (0.84), **G2** 10.00 (1.94), **G3** 7.70 (3.33), **G4** 5.80 (3.70), **G5** 7.20 (2.65). The statistical analysis showed significant differences between irrigants ($p < 0.05$) but not between root thirds ($p > 0.05$). The EDTA+NaOCl combination and polyacrylic acid showed greater capacity to remove the smear layer created during post preparation.

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Keywords: endodontics- smear layer- sodium hypochlorite- edetic acid.

Preparación para poste: limpieza lograda por diferentes protocolos de irrigación

RESUMEN

El objetivo del presente trabajo fue evaluar la capacidad de diferentes protocolos de irrigación para remover el barro dentinario de las paredes del conducto y entrada de los túbulos dentinarios, producido durante la preparación para anclaje intraradicular. Se utilizaron 50 premolares inferiores humanos a los cuales se les cortó la corona clínica. Luego se les realizó tratamiento endodóntico y obturación del tercio apical con cono único de conicidad aumentada y compactación vertical caliente. Se dejaron libres 10 mm para la preparación para poste que se realizó utilizando secuencialmente fresa de Largo #1 y fresa piloto Exacto #1 (Angelus, Brasil), irrigando a cada cambio de instrumento con agua destilada. Las muestras fueron distribuidas aleatoriamente en 5 grupos (n=10). G 1: No se efectuó ningún lavado. Los irrigantes fueron activados con cepillo mecánico cónico a baja velocidad. G 2: Agua destilada activada 60 s + agua destilada G 3: NaOCl al 5,25%, 15 s + agua destilada. G 4: EDTA al 17% (Farmadental, Argentina) 60 s + NaOCl al 5,25% 15 s, + agua destilada. G 5: Ácido poliácrico

al 10% (Densell) 15 s + agua destilada. Posteriormente, las raíces fueron seccionadas longitudinalmente en sentido vestibulo lingual. Cada superficie fue observada al MEB en sus diferentes tercios. Las micrografías se realizaron a 150 y 600 X y los resultados analizados estadísticamente mediante test de Kruskal Wallis y Friedman ($p < 0,05$). Los resultados [Medias (desviaciones estándar)] fueron: para 150X **G1** 11,00(1,33), **G2** 7,50 (3,13), **G3** 6,30 (2,58), **G4** 2,20 (2,80), **G5** 4,30 (1,50) y para 600X **G1** 11,40 (0,84), **G2** 10,00 (1,94), **G3** 7,70 (3,33), **G4** 5,80 (3,70), **G5** 7,20 (2,65). El análisis estadístico mostró diferencia significativa entre irrigantes ($p < 0,05$) pero no entre tercios radiculares ($p > 0,05$). La combinación de EDTA+NaOCl y el ácido poliácrico tendrían mayor capacidad de remoción del barro dentinario creado durante la preparación para poste.

Palabras clave: endodoncia- barro dentinario, hipoclorito de sodio, EDTA.

INTRODUCTION

Endodontically treated teeth usually have large carious lesions, crown fractures or overextended endodontic access that compromise retention of the restoration on the tooth remnant and therefore often require some form of anchoring for rehabilitation¹.

In the past, cast metal posts were used to connect the tooth remnant to the crown, but in recent years, fiber-reinforced resin based posts have appeared on the market. One of the most usual complications with this type of adhesively cemented posts is that they become detached, crack or leak. The most frequent failure is due to detachment of the post, since the tooth-resin interface tends to be the weakest point in these adhesive attachments²⁻⁵.

Different adhesive systems and resin cements are currently used for bonding fiber posts, among which self-conditioning-resin cements have become popular. This system is less sensitive to operatory technique, reduces the number of clinical steps and the risk of procedural errors that exist when other bonding systems are used (moisture control, adequate solvent evaporation, among others). Nevertheless, because it is a polymeric cement, it involves the same requirements regarding absolute isolation of the operatory field – essential during the bonding of rigid structures, particularly root posts, where root canal asepsis is a priority. Its chemical formula includes multifunctional monomers with groups derived from phosphoric acid, which simultaneously demineralize and infiltrate enamel and dentin. Although the material mainly hardens due to a polymerization reaction typical of resins, it is also believed that it adheres chemically to the calcium of the hydroxyapatite in the dentin, a concept which is applied in another group of materials – the glass ionomers⁶.

The main disadvantage of self-conditioning resin cements is their low capacity to demineralize and infiltrate the thick smear layer remaining on the walls of the root canal after post preparation^{6,7}.

The thickness of the smear layer in operative cavities is variable, ranging from 0.5 μm to 5.5 μm . It contains the same components as are present in dentin, such as collagen, glycosaminoglycans, denatured proteoglycans, hydroxyapatite, bacteria and minerals⁸. In post-endodontic dentin, it also contains odontoblastic processes and remains of endodontic filling material such as gutta-percha and endodontic sealer. The presence of the smear

layer can hinder the dentin-resin association, while removing it may promote adhesion.

Different chemicals are used in endodontics and operative dentistry to remove the smear layer from the cavity walls completely or partially, including phosphoric acid, citric acid, polyacrylic acid, EDTA and sodium hypochlorite, among others. Although 37% phosphoric acid used prior to cementing with Relyx U 200 (3M ESPE) has been used successfully on enamel, where tensile strength increases considerably, it produces a harmful effect on bonding in dentin. This may be due to the fact that it is difficult for self-conditioning cement to infiltrate the layer of collagen fibers without the mineral support provided by acid etching^{9,10}.

Action of 17% EDTA combined with sodium hypochlorite is based on EDTA chelating action on calcium ions, which produces gentle surface demineralization of dentin, while sodium hypochlorite acts subsequently, causing deproteinization of the collagen fibers denatured by the frictional heat in the area of burned or dehydrated dentin⁸. The use of sodium hypochlorite may have an adverse effect on bond strength, so it is controversial¹¹.

Different concentrations of sodium hypochlorite alone are also used as irrigants. However, although sodium hypochlorite alone thins the smear layer, it does not completely remove it. The smear layer is only removed completely when sodium hypochlorite is used in combination with a chelating agent, as described above¹¹⁻¹³, since sodium hypochlorite does not dissolve inorganic matter.

Polyacrylic acid is used prior to the application of glass ionomer cements. It is a weak acid, commercially available at concentrations of 10% to 25% in water, with variations in pH. At a concentration of 20%, its pH is 1, while at 10% it is 1.85. This difference in pH may be associated to its ability to clean dentinal walls¹⁴. Treatment of root dentin with polyacrylic acid prior to use of Relyx U200 may remove the smear layer, open dentinal tubules, create some tags and increase bond strength^{14,15}.

The aim of this study was to contribute to the knowledge on the points described above by evaluating the ability of different irrigation protocols to remove from canal walls and dentinal tubule entrances the smear layer produced as a result of preparation for a root post.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the Buenos Aires University School of Dentistry (code 001/2016).

This study used 50 human lower premolars, preserved in water at 37 °C until they were used, with average total length 21 mm to 23 mm, without carious lesions, with fully developed apex, a single straight canal and patency to catheterization. Teeth with excessively oval anatomy were excluded (as determined by X-ray showing that the vestibular-lingual:mesiodistal ratio in the middle third of the root canal was greater than 2:1).

In order to eliminate variables, clinical crowns were cut perpendicular to the long axis of the tooth, 2 mm above the proximal dentin-enamel junction, thus preserving a band of cervical dentin (ferrule effect). Then root catheterization was performed with a K#10 file (Dentsply Maillefer, Ballaigues, Switzerland). Working length was determined with a k #15 file at 1 mm before the apical foramen.

Root canals were prepared surgically using the ProTaper Universal mechanized instrumentation system (Dentsply Maillefer, Ballaigues, Switzerland), which consists of six nickel-titanium instruments with variable taper, according to the manufacturer's instructions. Specimens were instrumented using an electric motor with controlled torque at 300 rpm (X Smart Plus, Dentsply Maillefer, Ballaigues, Switzerland), irrigation was performed after every change of instrument by applying 2 ml of 2.5% sodium hypochlorite solution with Terumo 25G1 syringe and needle, up to a final instrument F3(30/09).

Final irrigation was performed with 17% EDTA (Farmadental, Buenos Aires, Argentina) for 1 minute, after which the root canal was dried with absorbent paper points, then rinsed with 2 ml of 2.5% sodium hypochlorite solution for 30 s. Both irrigant solutions were agitated with a sonic device with polymer tips (Endoactivator, Dentsply Tulsa, USA). Then the root canals were dried with absorbent paper points and the apical third filled with a F3 ProTaper gutta-percha point (Dentsply Maillefer, Ballaigues, Switzerland) followed by warm vertical compaction with Calamus Dual system (Dentsply, Maillefer). AH Plus (Dentsply, DeTrey, Germany) was used as endodontic sealer. This filling technique was used in order to keep free from endodontic material the walls of the cervical and middle thirds of root canal,

where the space for the root post would be shaped. Post space was prepared at the same operatory time using #1 Maillefer Largo drills (Dentsply Maillefer, Ballaigues, Switzerland) and #1 calibrator drill white ring (#70 apical diameter) from the kit (Exacto 1, Angelus, Brazil). Post space length was established at 10 mm from the cervical edge. During the procedure, irrigation was performed with distilled water to promote smear layer removal, lubricate and cool the root canal. Post space cleanness was evaluated using a stereomicroscope (Newton, Buenos Aires, Argentina) at 16X magnification. Specimens were divided randomly into 5 groups (n=10) with different irrigation regimens (see below). All except the negative control group were irrigated using a Terumo 25 G1 syringe and needle (Terumo, Argentina) with rubber stopper placed 10 mm deep, with the needle moving gently in and out, allowing free backflow of irrigant solutions, and activated with a Rotoprox conical mechanical brush (HagerWerken, Germany) at low speed. Specimens were labelled according to the experimental groups listed below.

Group 1 (negative control): No irrigation after post preparation.

Group 2: Irrigation with **distilled water** (3 ml) activated with Rotoprox for 60 s and final rinse with distilled water (6 ml).

Group 3: Irrigation with 5.25% **sodium hypochlorite** solution (3 ml) activated with Rotoprox for 15 s and final rinse with distilled water (6 ml).

Group 4: Irrigation with 17% **EDTA** solution (Farmadental, Buenos Aires, Argentina) (3 ml) activated with Rotoprox for 60 s, followed by 5.25% **sodium hypochlorite** solution (3 ml) activated with Rotoprox for 15 s and final rinse with distilled water (3 ml).

Group 5: Irrigation with 10% **polyacrylic acid** solution (Densell) (3 ml) activated with Rotoprox for 15 s and final rinse with distilled water (6 ml).

Teflon and a thin layer of temporary filling material (Coltosol F, Coltene, Switzerland) were placed inside the canal to prevent entry of foreign particles and keep the samples immersed in water at 37 °C.

Two deep longitudinal grooves were shaped on the free faces using a constantly water-cooled low-speed diamond disc, without reaching the root canal lumen. Then the teeth were split in half using an ad-hoc clamp. A fine-tip marker was used to mark the sides of each canal at 2 mm, 5 mm y 8 mm, in order

to enable identification of the points to be observed under scanning electron microscope.

The samples were dehydrated, mounted on an aluminum base, coated with a 20 nm gold-palladium layer and observed under scanning electron microscope (Zeiss, model Supra 40). Micrographs were taken at magnifications of 150X and 600X of the middle area of each third, at 2 mm (coronal third), 5 mm (middle third) and 8 mm (apical third) from the coronal reference.

The following numerical categories were used to evaluate cleanness of the root dentinal substrate:

Scale for cleanness (150X magnification)

0: No smear layer.

1: Some particles with less than 20 µm across.

2: A lot of smear layer.

Scale for dentinal tubules (600X magnification)

0: All dentin tubules open with no visible smear layer.

1: Some dentinal tubules open and a thin smear layer covering tubule entrances.

2: All dentinal tubules covered.

The sum of the scores per category recorded for total observations performed on each experimental unit was taken as indicator for the situation of the variables (cleanness and tubules). Kruskal Wallis test was used for comparison among groups. For comparison of thirds within each group, the indicator considered was the sum of scores observed for each, and Friedman's test was applied. When required, tests for multiple comparisons were used. Significance was set as $p < 0.05$ for all cases.

RESULTS

Table 1 shows the results for the different groups, with arithmetic mean and standard deviation of the sum of scores for the variables cleanness and tubule exposure. Mean values for dentinal tubule opening were lowest (most open) in Group 4 (EDTA + sodium hypochlorite) and Group 5 (polyacrylic acid), though without significant difference compared to Group 3 (sodium hypochlorite). The 600x scanning electron microphotographs show that in Group 3 (sodium hypochlorite), the smear layer on dentin walls is thinner than in Group 2 (distilled water), although there is detritus plugging the lumen of dentin tubules. Group 4 (EDTA + sodium hypochlorite) and Group 5 (polyacrylic acid) are cleaner, with a higher number of open tubules (Fig. 1). Root walls were cleaner in Group 4 (EDTA +

sodium hypochlorite) though not significantly different from Group 5 (polyacrylic acid). The 150X microphotographs show that brushing with water only (Group 2) was insufficient to clean the root dentin surface completely. Chemical solutions used as irrigants achieved greater cleanness (Fig. 2).

Practically no difference was observed for cleanness between root thirds, and when there was, the middle third was the cleanest (Table 2).

DISCUSSION

It is important to highlight that this study compared irrigation protocols, i.e., procedures to be performed by the operator before luting a root post with resin cement. These steps are relevant when using self-conditioning resin cements which do not require prior treatment of the dentinal substrate.

By definition, a procedure consists of following certain predefined steps in order to perform a task efficaciously. There may be different procedures for the same purpose, each of which will involve different steps and be more or less efficient.

The different activation times for the irrigants in each group are related to the specific intended chemical action on the smear layer, considering that brushing will exert mechanical action on it at the same time.

This study compares the effects of different irrigant solutions. From a clinical standpoint, it seeks to identify which solution achieves the cleanest possible dentin substrate in the shortest time and the simplest manner, with the aim of avoiding further complication of the maneuvers within the root canal. The procedures were selected based on the literature and were not standardized. Different irrigation protocols were found to provide different levels of cleanness and tubule opening.

Table 1. Results (arithmetic means and standard deviations) for cleanness and tubule exposure for each experimental group.

Group	Cleanness	Tubules
I	18.6 (1.9) c	19.0 (1.5) c
II	12.1 (5.0) b	16.6 (3.1) b,c
III	10.6 (4.5) b	12.6 (5.5) a,b
IV	3.3 (4.2) a	8.7 (6.6) a
V	6.8 (2.3) a,b	11.7 (4.6) a,b

No statistically significant difference ($P > 0.05$) between values identified with the same letter within each variable.

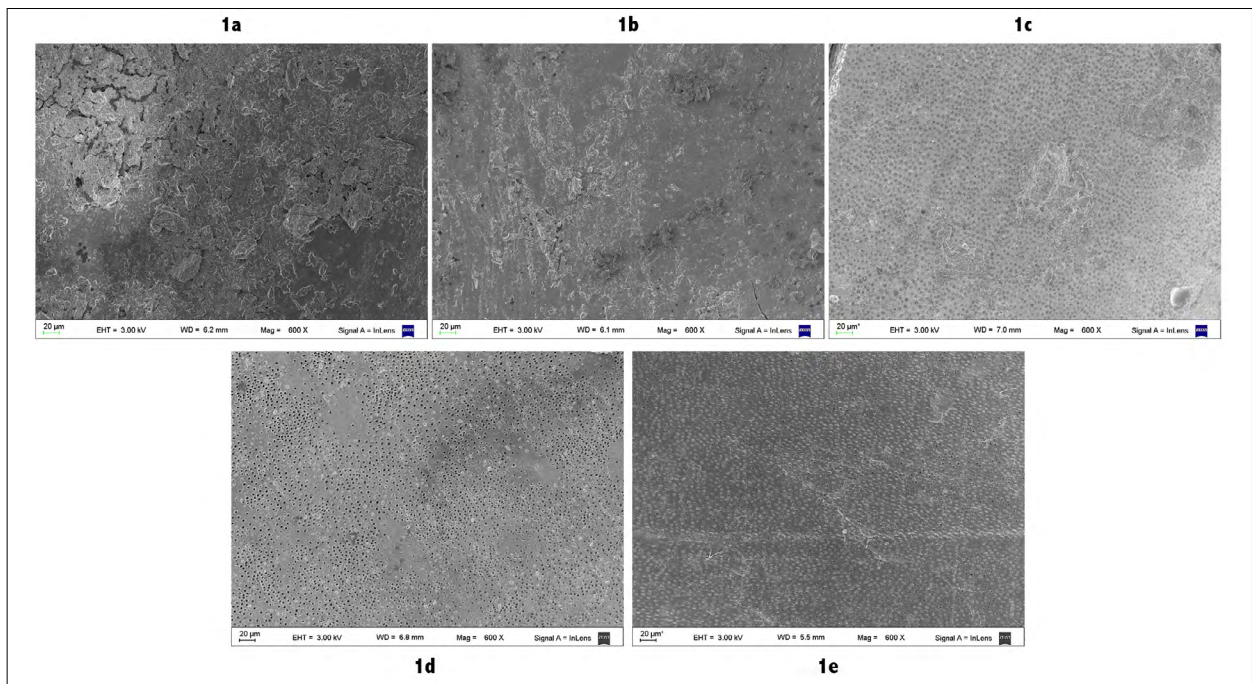


Fig.1. Scanning electron microphotographs of root middle third (600X) for observation of dentinal tubule openings.

1a: Group 1, without irrigation. There is a dense, irregular smear layer completely covering the dentinal surface. 1b: Group 2, irrigation with distilled water activated with brushing. Persistent smear layer covering entrance to dentinal tubules. 1c: Group 3, irrigation with sodium hypochlorite. Noticeable thinning of smear layer, showing many dentinal tubules plugged with detritus. 1d: Group 4, irrigation with EDTA+ sodium hypochlorite. Many exposed dentinal tubules with opening free, without detritus on the surface. 1e: Group 5, irrigation with polyacrylic acid. Clean, many exposed dentinal tubules, though most of them are plugged.

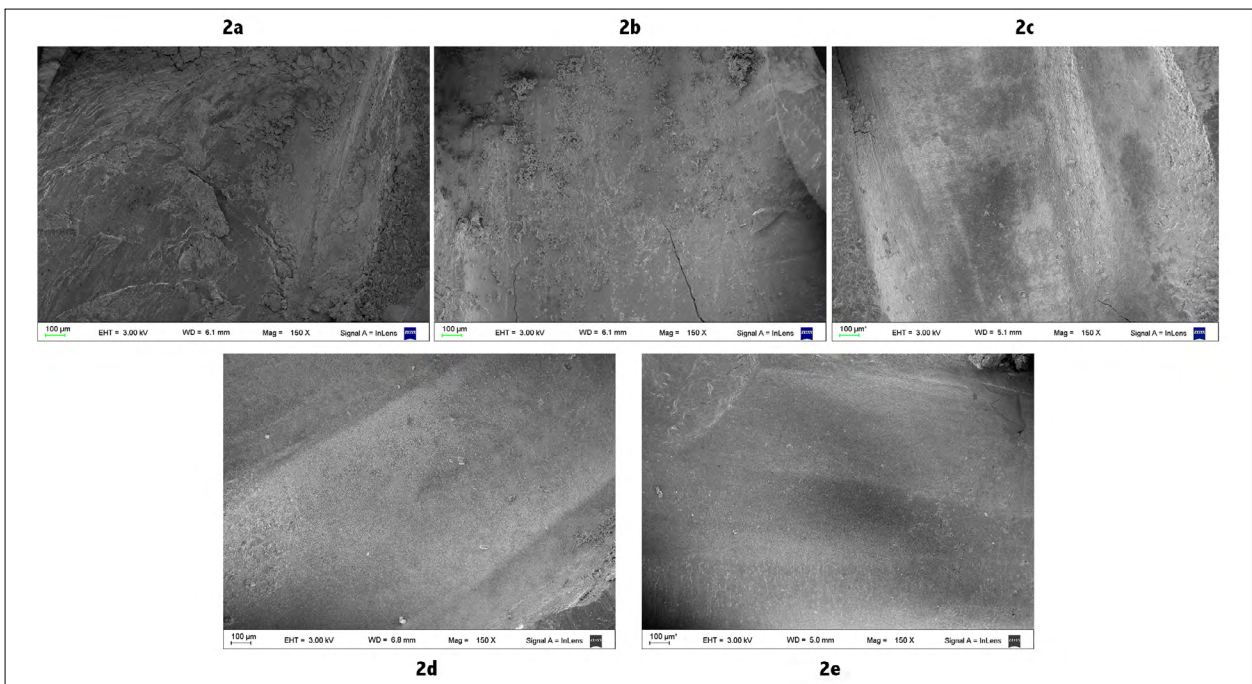


Fig. 2. Scanning electron microphotographs of the root middle third (150X) for observation of cleanliness of root dentin surface.

2a: Group 1, without irrigation. There is a thick, dense, irregular smear layer with large detached particles greater than 100µm across. 2b: Group 2, irrigation with distilled water activated with brushing. There is a thinner smear layer, adhered to the surface, with smaller loose particles, less than 100µm across. 2c: Group 3, irrigation with sodium hypochlorite. There is a thinned smear layer without deposited particles. 2d: Group 4, irrigation with EDTA+ sodium hypochlorite. There is a clean dentinal surface, with few areas covered in detritus. 2e: Group 5, irrigation with polyacrylic acid. Cleanliness similar to Group 4.

Table 2. Comparison of cleanness and tubules between thirds within each experimental group.

Group	Cleanness			Tubules		
	Middle	Cervical	Apical	Middle	Cervical	Apical
I	1.2 a	2.6 b	2.3 b	2.0 a	2.1 a	2.0 a
II	1.4 a	2.1 b	2.6 b	2.0 a	1.8 a	2.2 a
III	1.3 a	2.4 b	2.3 b	1.4 a	2.3 b	2.3 b
IV	1.8 a	2.0 a	2.3 a	1.5 a	2.9 b	2.7 b
V	1.6 a	2.1 b	2.4 b	1.8 a	1.9 a	2.3 a

Values are means of the ordinal number for the third within each group and variable. Same letter within the third and the variable indicates non-significant difference ($P>0.05$).

Irrigation with distilled water plus mechanical brushing was insufficient to clean the root dentin surface. Groups 4, 5 and 3 do not differ significantly from each other regarding the effect on dentin, though it should be noted that Group 4 tended to have better results. The lack of a noticeable statistical difference among groups may be due to the impossibility of homogenizing and evaluating the initial characteristics and cleanness of the root dentin. Some variables that are reflected by dentinal structure which are very difficult to control, such as tooth age, bruxism or other habits which may promote data dispersion.

In this *ex vivo* study, observations were made under scanning electron microscope at 150X and 600X. The lower magnification (150X) provides a larger field of view, enabling a more general view for analyzing amount of smear layer and detritus, while the higher magnification (600X) enables identification of more sensitive situations such as removal of the smear layer on intertubular dentin and presence of plugs in dentinal tubule lumens.

A conical Rotoprox brush was used at low speed throughout the irrigant action period in an attempt to improve mechanical removal of the smear layer and detritus and to increase the contact between the irrigant and the dentin substrate. In a comparative study of cleaning preparation for root posts, Goldberg et al. reported that brushes soaked in alcohol did not improve the cleanness achieved with the original preparation¹⁶. The fillings-in said study were performed using hybrid technique (thermomechanical compaction), which probably hindered full removal of material stuck to canal walls.

Taking this into account, in the present study, post preparation was performed during the same endodontic treatment session by filling only the

apical third and leaving the middle and coronal thirds free from endodontic material. When thirds were compared for cleanness, the middle third was found to be cleaner, possibly due to greater contact of the conical brush against the root canal walls.

Except for polyacrylic acid, the irrigants used in this study are commonly used in endodontic procedures. EDTA at 17% acts as a chelating agent of calcium ions, producing surface demineralization of dentin. Sodium hypochlorite at different concentrations causes deproteinization of exposed collagen fibers. This is probably why overall, Group 4 had the best results, in addition to being the irrigation protocol that used the Rotoprox brush for the longest time, perhaps achieving greater mechanical removal of the smear layer. This effect was also observed by Wu et al., who report that a smear layer of approximately 5 μm generated by post preparation was practically dissolved after using EDTA and sodium hypochlorite as irrigants, with dentinal tubule orifices becoming visible¹⁷. Several authors have confirmed that the use of EDTA for long time causes mineral loss in intertubular and peritubular dentin, thereby reducing dentin hardness, so they recommend that exposure to this chelating agent should not exceed 5 minutes. Excessive exposure to EDTA may cause areas of eroded dentin, and this was also observed in some of the microphotographs in the present study. These results are in agreement with Ensinas et al. and other papers^{18,19}.

In the present study, the smear layer was cleaned less in the group irrigated only with 5.25% sodium hypochlorite solution than when it was combined with EDTA, even though the layer was thinned, in agreement with Gu et al. In that study they report that the smear layer was mainly removed from the middle and coronal thirds of the preparation, although even so, dentinal tubules remained blocked with

detritus²⁰. Guerisoli et al. report that the action on the smear layer of a 1% sodium hypochlorite solution was similar to that of distilled water²¹. Baumgartner et al. report that 5.25% sodium hypochlorite in combination with ultrasonic agitation instead of rotary brushes did not effectively remove smear layer from root canal walls²².

It is worth noting that the manufacturer's instructions for Relyx U200 (3M) self-conditioning resin cement call for pre-treatment of root dentine by irrigation with sodium hypochlorite followed by rinsing with water. This recommendation may be related to the material's low affinity with the hydrated collagen fiber layer. When the organic components of the smear layer are removed, the mineral component increases and water content decreases. Relyx U200 contains functional phosphoric methacrylates that react with the calcium ions of the hydroxyapatite²³. The current study used 5.25% sodium hypochlorite for 15 s because exposure times of 30 s or longer would affect resin cement polymerization²⁴.

The third chemical compared in the present study was polyacrylic acid, which is used prior to applying

glass ionomer cements in order to achieve greater interaction between the material and the dentin substrate²⁵.

In the current study, the action of 10% polyacrylic acid for 15 s proved to be sufficient to clean the dentin walls, with no significant difference compared to the EDTA/hypochlorite combination. Mazitelli et al. verified removal of the smear layer, opening of dentinal tubules and presence of some tags when dentin was pre-treated with polyacrylic acid prior to use of Relyx U200, and Stona et al.^{15,14} found an increase in the bond strength of Relyx U200 and resin tag formation following pretreatment with 11% polyacrylic acid. These aspects were not evaluated in the present study.

CONCLUSIONS

Significant differences were found between irrigation protocols, though differences between root thirds were not very marked. Both the EDTA+NaOCl combination and polyacrylic acid appear to have the greatest ability to remove the smear layer during post preparation.

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Melatonin has a stimulatory effect on osteoblasts by upregulating COL-I and OPN expression/secretion

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ABSTRACT

Melatonin (MLT) is a potential signaling molecule in the homeostasis of bone metabolism and may be an important mediator of bone formation and stimulation. The aim of this in vitro study was to evaluate the effect of MLT on the viability, mRNA/protein expression and mineralization of pre-osteoblastic cells. The concentrations 5, 2.5, 1, 0.1 and 0.01 mM MLT were tested on pre-osteoblastic cells (MC3T3) compared to control (no MLT), evaluating proliferation and cell viability (C50), gene expression (RT-PCR) and secretion (ELISA) of COL-I and OPN at 24h, 48h and 72h, and the formation of mineral nodules (alizarin red and fast red) after 10 days of treatment. MLT at 5 and 2.5 mM proved to be cytotoxic (C50), so only 0.01, 0.1 and 1 mM were used for the subsequent analyses. OPN mRNA

expression increased with MLT at 0.1 mM - 1 mM, which was followed by increased secretion of OPN both at 24h and 72h compared to the remaining groups ($p < 0.05$). COL-I mRNA and COL-I secretion followed the same pattern as OPN at 0.1 mM MLT at 72h of treatment ($p < 0.05$). Regarding mineralization, all MLT doses (except 1mM) caused an increase ($p < 0.05$) in the formation of mineral nodules compared to the control. Melatonin at 0.01mM - 1mM had a stimulatory effect on osteoblasts by upregulating COL-I and OPN expression/secretion and mineralization, thereby fostering osteogenesis.

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Keywords: collagen- melatonin- osteogenesis- osteopontin.

Melatonina tem efeito estimulador em osteoblastos, pela regulação positiva da expressão/secreção de COL-I e OPN

RESUMO

A melatonina (MLT) é uma molécula potencial de sinalização na homeostase do metabolismo ósseo e pode ser um importante mediador da formação e estimulação óssea. O objetivo deste estudo in vitro foi avaliar o efeito da MLT na viabilidade, na expressão do mRNA da proteína e mineralização de células pré-osteoblásticas. As concentrações de MLT 5, 2,5, 1, 0,1 e 0,01 mM foram testadas em células pré-osteoblásticas da linhagem MC3T3 em comparação ao controle (sem MLT), avaliando a proliferação e a viabilidade celular (C50), expressão gênica (rtPCR) e secreção (Elisa) de Colágeno tipo 1 (COL-I) e osteopontina (OPN) às 24, 48 e 72 horas, além da formação de nódulos minerais por meio do teste vermelho de Alizarina fast red após 10 dias de tratamento. MLT a 5 e 2,5 mM provou ser tóxico (C50). Portanto, as concentrações de 0,01, 0,1 e 1 mM

foram utilizadas para as análises subsequentes. A expressão do mRNA da OPN aumentou com MLT a 0,1 mM-1mM, seguida pela secreção aumentada de OPN às 24 e 72 horas em comparação aos demais grupos ($p < 0,05$). O mRNA de COL-I e a secreção de COL-I seguiram o mesmo padrão do OPN a 0,1 mM de MLT em 72 horas de tratamento ($p < 0,05$). Em relação à mineralização, todas as doses de MLT (exceto 1mM) causaram aumento ($p < 0,05$) na formação de nódulos minerais em comparação ao controle. A MLT na concentração entre 0,01mM a 1 mM teve um efeito estimulador sobre os osteoblastos, ao regular positivamente a expressão e secreção de COL-I e OPN, além da mineralização, favorecendo a osteogênese.

Palavras-chave: colágeno- melatonina- osteogênese- osteopontina.

INTRODUCTION

Melatonin (MLT) is the main hormone synthesized and secreted by the pineal gland. Its functions include regulating various physiological processes

in different parts of the body, such as circadian rhythm¹, body temperature², immune system³, sexual development⁴ and reproductive cycle⁵. MLT levels in the body are related to ambient light, reaching

maximum levels at night when it is dark and lowest levels throughout the duration of daylight⁶.

MLT may be administered in several ways such as orally and intraperitoneally as a supplement or prescription-only, depending on the country. It is inexpensive and has few side effects compared to other drugs and can be used with a wide safety margin. Such features combined with the series of reported effects on different tissues make melatonin a potent therapeutic option for several ailments and/or prevention thereof⁷.

Studies have demonstrated the influence of MLT in cell differentiation by stimulation or suppression, depending on dose and cell type^{8,9}. MLT is believed to have the potential to play a key role in regulating bone growth since at therapeutic doses it may inhibit osteoclastic activity and induce osteoblast differentiation¹⁰.

A previous study revealed that MLT enhances mesenchymal stem cell differentiation into mature osteoblasts through the MT2 receptor¹¹ and during osteoblastic differentiation, MLT increases the expression of osteocalcin (OC), a highly specific marker of bone formation, followed by enhanced bone mineralization¹². It has also been demonstrated that MLT has the ability to stimulate the proliferation and synthesis of type I collagen, as well as other bone matrix proteins and bone markers (alkaline phosphatase, osteopontin and osteocalcin) at micromolar concentrations in human osteoblasts *in vitro*⁹, though the mechanisms by which such phenomena take place remain largely ill-understood. The oral cavity is affected by a number of conditions such as periodontitis, mucositis, neoplasms and cytotoxicity from various drugs and/or biomaterials. MLT has been shown to down-regulate the expression of pro-inflammatory factors such as C-reactive protein, interleukin-6 and tumor necrosis factor- α ² as well as receptor activators of nuclear factor kappa-B ligand/osteoprotegerin ratios to reduce periodontal inflammation^{2,13,23,24}. In addition to up-regulating salivary acid phosphatase, alkaline phosphatase, osteopontin, and osteocalcin, MLT has been associated to positive outcomes regarding gingival index and pocket probing depth, which are highly suggestive of enhanced osteoblast differentiation and bone formation^{14, 17,23,24}.

Despite the numerous current and potential applications of MLT, further studies are needed to ascertain the effect of melatonin on bone cells to

serve as the basis for *in vivo* studies and ultimately safe long-term clinical use. Therefore, the aim of the present study was to evaluate *in vitro* the effect of the hormone melatonin on the proliferation, viability, bone matrix protein expression and mineralization capacity of pre-osteoblastic cells.

MATERIALS AND METHODS

Experimental design

This was an analytical bifactorial experimental study, in which the following doses of melatonin were tested: 0.01mM (C1), 0.1mM (C2), 1mM (C3), 2.5mM (C4) and 5mM (C5) and a control group (C6) at 24h, 48h, 72h and 10 days after treatment.

MLT powder $\geq 98\%$ (N-acetyl-5-methoxytryptamine) and M5250 Sigma (Sigma, St. Louis, Missouri, USA), reconstituted in 50 mg/mL absolute ethanol were used. The control group received the highest dose of ethanol (vehicle), but no MLT.

Cell culture

A mouse-derived pre-osteoblastic cell line (MC3T3-E1) obtained from the ATCC (American Type Culture Collection, ATCC, VA, USA) was used, supplemented with 15% fetal bovine serum (Nutricell Cellular Nutrients, Campinas, SP, Brazil), 100 IU/ml penicillin (Sigma) and 50 mg / ml streptomycin (Sigma).

For the mineralization assays, the cultures were supplemented with 50 μ g/mL ascorbic acid, 100 μ g/mL beta-glycerophosphate (Sigma, St. Louis, MO, USA), 95% O₂ and 5% CO₂.

At 70% sub-confluence, the culture medium was removed and 0.25% trypsin (Nutricell Nutrientes Celulares) and 1mM EDTA (Gibco) solution were added to gather the cells in suspension. Then the cells were seeded - 3.5x10³ cells / well in 96-well polystyrene plates (Sarstedt). After 24 hours, the medium was changed and the osteoblastic cells treated with MLT at 0.01, 0.1, 1, 2.5 and 5 mM.

Cell proliferation assay

The Trypan blue vital exclusion method was used at 24h, 48h and 72h of treatment with MLT at 0.01, 0.1, 1, 2.5 and 5 mM.

Cell viability assay (MTT)

Cell cultures were tested for cell viability using the MTT assay. After solubilization of the crystals,

quantification was performed on an ELX800 microplate reader (Biotek Instruments, Inc.) at 590 nm. The cytotoxic dose (C50) was then determined based on the MTT assay findings. This represents cell viability below 50% in relation to the control group.

mRNA expression for collagen type I (COL-I) and osteopontin (OPN)

Real-time polymerase chain reaction (RT-qPCR) was used to evaluate the transcription levels of genes encoding COL-I and OPN proteins. Table 1 shows the sequence of primers.

At 24h, 48h and 72h, the culture medium was removed from the wells (stored at -80°C for secreted protein assays) and the cells were collected for RNA extraction using the Illustra RNASpin Mini Kit (GE Healthcare, Milwaukee, WI, USA), according to the manufacturer's instructions. Samples were quantified by spectrophotometry (NanoVue) and the quality was established at A260/A280 > 1.6. The complementary DNA was synthesized using the ThermoScientific cDNA kit, according to the manufacturer's instructions.

The reactions of were performed using the SYBR Green system (Thermo Fisher Scientific, Waltham, MA, USA) on a Real Time PCR System (Applied Biosystems, Foster City, CA, USA).

The results were analyzed based on Ct (Cycle Threshold), which corresponds to the number of cycles at which the amplification of the samples reached a threshold between the fluorescence level and the amplification phase (determined between the fluorescence level in relation to the negative control, C6). The 2-ΔΔCT method (Livak & Schmittgen, 2001) was used to determine expression of the target genes.

Total protein assay

Prior to the protein secretion assays (ELISA), the culture medium was normalized via total protein quantification following the method of Lowry et al. (1951), in a spectrophotometer (CE3021, Cecil, Cambridge, England) at 680 nm. Total protein concentration was calculated for each well in g/mL from a standard curve based on bovine serum albumin (Sigma).

Enzyme immunoassay for collagen type I (COL-I) and osteopontin (OPN)

Quantification of COL-I and OPN secreted by the osteoblastic cells under different MLT doses was

Table 1. Sequence of primers for collagen type I (COL-I), osteopontin (OPN) and the endogenous control (GAPDH).

Gene name	Gene symbol	Primer Sequence
COL- I	<i>COL1A1</i>	F 5' CCAGAAGAACTGGTACATCAGCAA-3' R 5' GGACATCAGGCGCAGGAA-3'
OPN	<i>OPN</i>	F 5'-TGCTTGGGTTTG-CAGTCTTCT-3' R 5'-CCAAACAGGCAAAAGCAAATC-3
Glucose 6 phosphate dehydrogenase	<i>GAPDH</i>	F 5' ACCCACTCCTC-CACCTTTGA-3' R 5'-TGTTGCTGTAGC-CAAATTCGTT-3'

performed using ELISA (Kit ELISA for COL-I R&D Systems, EUA; Kit ELISA for OPN Cloud-Clone Corp, Katy, TX, USA).

The reactions were terminated by adding 50 µL of 2N sulfuric acid (H₂SO₄) to the substrate solution in each well and the color was measured on a spectrophotometer (Epoch, Biotek, Winooski, VT, USA) at 450 nm. The total amount of COL-I and OPN were determined in picograms per milliliter (pg/mL). All experiments were performed in triplicate. The primers used are listed in Table 1.

Mineralization assays

Alizarin red

Osteoblastic cells, except those from the control group, were cultured with MLT at 0.01, 0.1 and 1 mM and analyzed at 10 days and 14 days, with the culture medium changed every 2-3 days together with supplementation of MLT. The wells were rinsed and prepared for evaluation under a light microscope (Nikon eclipse E800, Japan). The presence of calcium deposits was identified by the development of red color in the matrix and photographs were taken. The images were processed and later analyzed on Image J (NIH). The percentage of calcium nodules formed was determined based on optical density from the images taken from each sample.

After 10 days of culture with the different concentrations of MLT, 280 µl of 10% acetic acid was added to each well and the plate was shaken for 30 min. The cell layer was then scraped off and the solution transferred to 1.5 ml tubes, which were vortexed for 30 s. Samples were first heated at 85 °C for 10 min and immediately placed in ice for 5 min. Subsequently, the tubes were centrifuged at

13,000g for 20 min and 100 µl of the supernatant was transferred to a 96-well plate, where 40 µl of 10% ammonium hydroxide was added to each well. The samples were read on a spectrophotometer at 405 nm.

Alkaline phosphatase assay - Fast Red test

The cultures were treated as per the alizarin red assay until the culture medium was removed and the wells washed with Hanks' solution (Sigma) heated to 37 °C. Three hundred and twenty milligrams of the Triz reagent (Sigma) were dissolved in 20 mL of deionized water for further addition of 7 mg of the Fast Red Reagent (Sigma). Two mL of this solution were discarded and 8mg Naftol (Sigma) diluted in 2mL of dimethyl formamide (Merck), forming the working solution. One ml of this solution was added to each well and the plate was placed in a moist-air incubator at 37 °C with 5% CO₂ for 30 min. The solution was then withdrawn and the plate dried for further photographic documentation. The images were processed and analyzed on Image J (NIH), which determined the percentage of nodules formed as a function of the amount of black areas within the total field.

Statistical analysis

Data were analyzed using two-way analysis of variance and multiple comparisons were performed using the Tukey test. The analyses were performed on Graphpad Prism version 6.0, considering a significance level of 5%.

RESULTS

Cell proliferation assay

Time and concentration factors significantly influenced cell proliferation ($p < 0.05$), with a significant increase in proliferation over time in relation to the control group and at 0.01 mM and 0.1 mM MLT. From analysis of the data at 24h, no differences were observed between the doses evaluated ($p > 0.05$). At 48h and 72h, higher mean proliferation values were observed with the lower doses (0.01, 0.1 and 1 mM). Multiple comparisons showed differences, especially at 48h and 72h, in which the higher doses (2.5 and 5 mM) caused a significant decrease in cell proliferation ($p < 0.05$). This finding guided the exclusion of the doses 2.5 mM and 5 mM from the PCR, ELISA and mineralization assays (Fig.1).

Cell viability assay (MTT)

The data relating to the MTT assay demonstrated a very similar pattern to that from the proliferation assay in terms of both dose and time, as well as the interaction between the two factors. As shown in Fig. 2, it was observed that at 24h, the lowest mean cell viability occurred at 5 mM MLT ($p < 0.05$). At 48h, the mean cell viability was observed at 5 mM, which was significantly lower than for 0.1 mM ($p < 0.05$). At 72h, both 5 mM and 2.5 mM showed a significantly lower mean cell viability than the remaining groups ($p < 0.05$). At 72h, the mean viability was significantly higher than at 24h at the doses of 0.01, 0.1 and 1mM and the control. The fact that the higher concentrations (2.5 and 5 mM) caused a significant decrease in cell viability also led to their exclusion from the subsequent analyses.

Alizarin Red Assay

At 10 days, as shown in Table 2 and Fig. 3, all doses caused a significant increase ($p < 0.05$) in the formation of mineral nodules when compared to the control group.

The 0.1 mM dose caused the highest quantity of mineral nodules ($p < 0.05$; Fig. 4). The remaining doses did not differ significantly from the control group, with 1 mM showing the lowest quantity of mineral nodules. As shown in Table 2 and Fig. 5, a significant increase in mineral nodules ($p < 0.05$) is observed only for the 0.1 mM group.

Gene expression of COL-I and OPN

At 24h, the 1 mM dose significantly up-regulated COL-I gene expression when compared to the remaining groups ($p < 0.05$). For OPN, a significant up-regulation occurred with the dose 0.1 mM ($p < 0.05$).

At 48h, COL-I expression was significantly up-regulated for 0.1 and 1 mM ($p < 0.05$), while OPN expression was significantly increased in the 0.01 mM and 1 mM groups ($p < 0.05$).

At 72h, COL-1 expression was significantly up-regulated in 0.1 mM group ($p < 0.05$) whereas OPN expression peaked at 1mM ($p < 0.05$, Fig. 6).

For the factor "time", COL-I expression was highest at 72h for the doses 0.1 mM and 1 mM. This also occurred for OPN expression at 1 mM ($p < 0.05$, Fig. 7).

COL-I and OPN secretion (ELISA)

The 1 mM dose of MLT significantly increased COL-I secretion when compared to the remaining

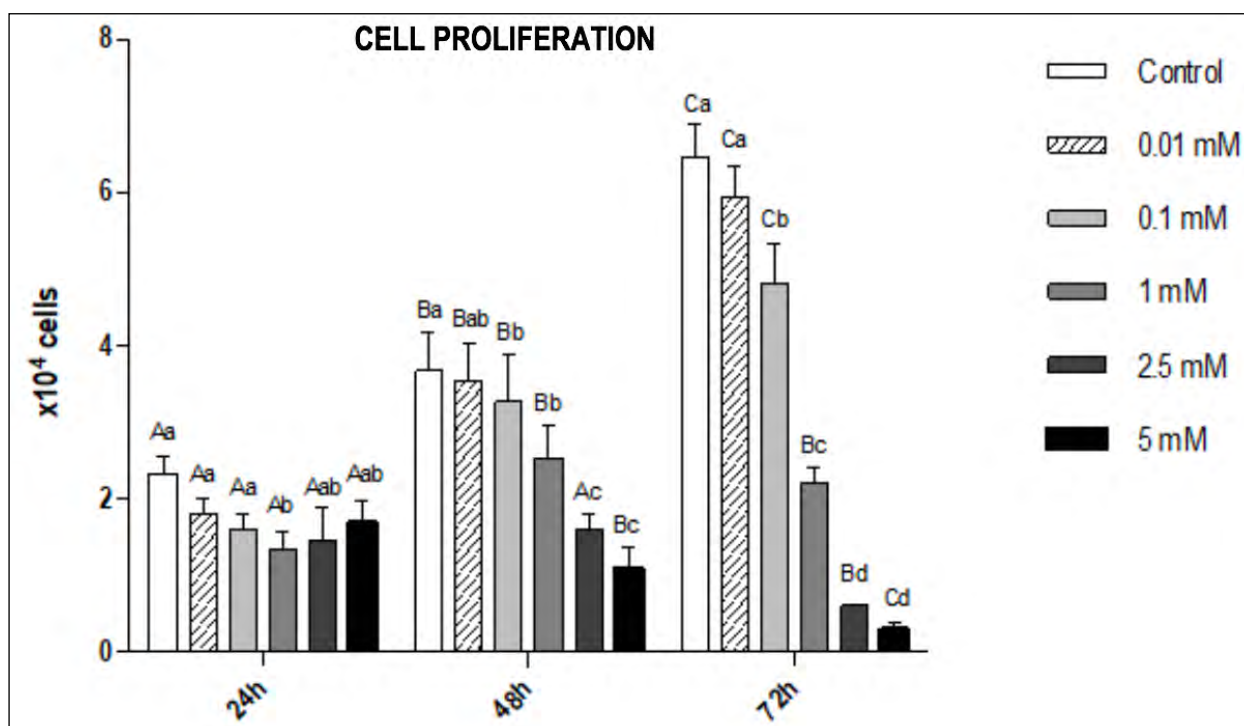


Fig. 1. Evaluation of cell proliferation by the Trypan blue vital exclusion method in pre-osteoblastic cells at 24h, 48h and 72h, supplemented with MLT at 0.01, 0.1, 1, 2.5 and 5 mM, and the control group. Different uppercase letters represent a significant difference between the evaluation periods for each studied condition (ANOVA and Tukey, $p < 0.05$); different lowercase letters represent a significant difference between the concentrations tested (ANOVA and Tukey, $p < 0.05$).

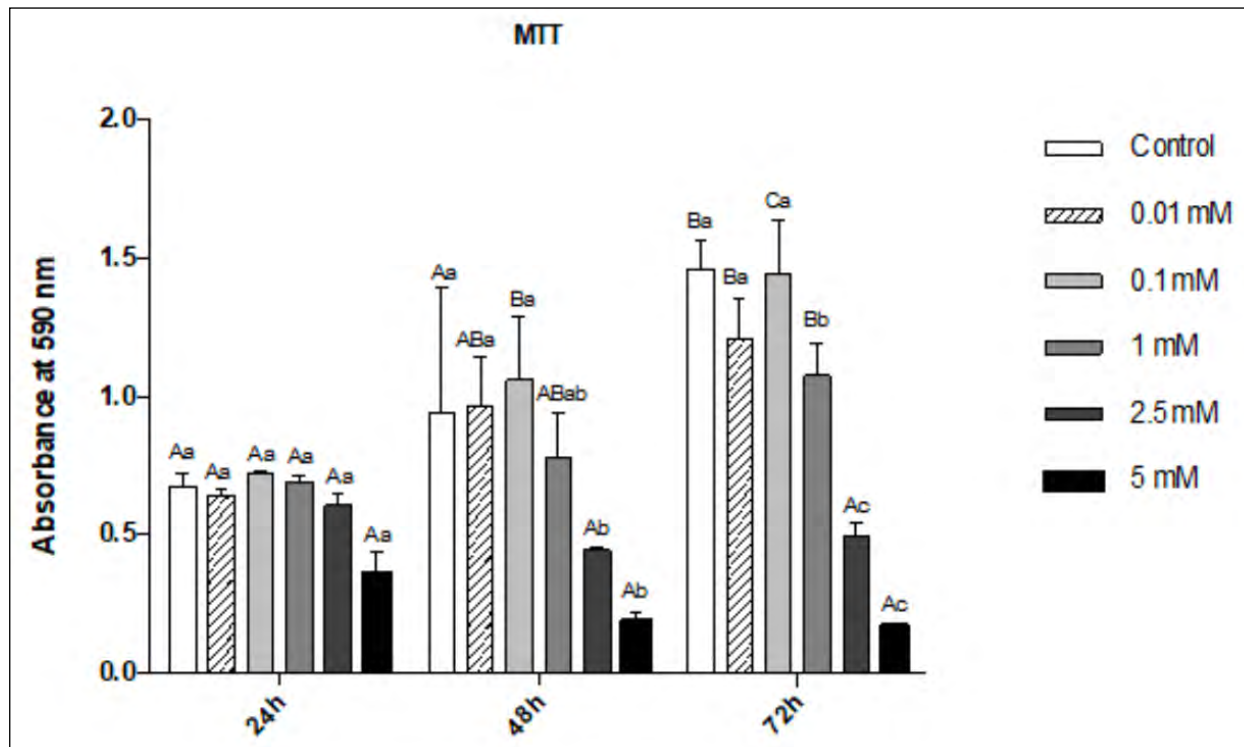


Fig. 2. Cell Viability Assay (MTT) in pre-osteoblastic cells at 24h, 48h and 72h, supplemented with MLT at 5, 2.5, 1, 0.1, 0.01 mM and the control group. Different uppercase letters represent a significant difference between the evaluation periods for each studied condition (ANOVA and Tukey, $p < 0.05$); different lowercase letters represent a significant difference between the concentrations tested (ANOVA and Tukey, $p < 0.05$).

Table 2. Means (standard deviation) of 10-day mineral nodule counts evaluated using the Alizarin Red (VA) and Fast Red (FR) tests.

	CRTL	0.01 mM	0.1 mM	1 mM
VA	15.35 (1.88) A	29.93 (6.03) B	68.24 (3.82) C	45.43 (1.45) D
FR	42.42 (1.93) A	29.43 (5.36) B	66.17 (2.46) C	14.64 (1.31) D

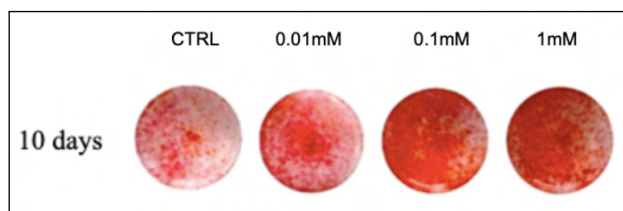


Fig. 3. Representative images from the Alizarin Red test at 10 days, for the different MLT concentrations studied.

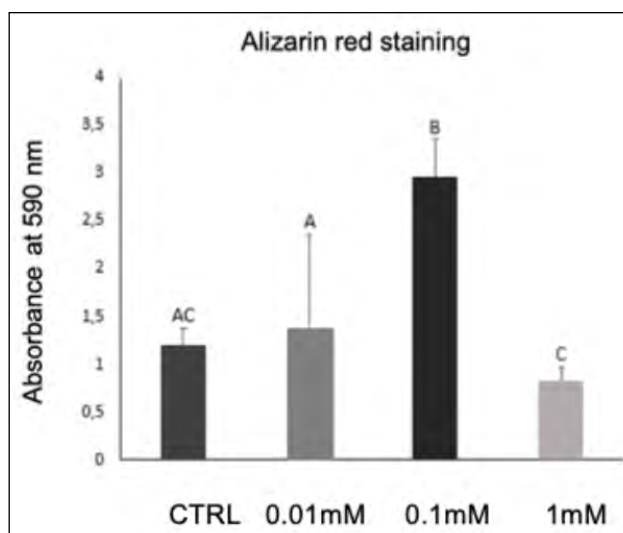


Fig. 4. Mean and standard deviation of Alizarin Red staining for the different MLT concentrations studied at 10 days. Different letters in the columns represent a significant difference between doses (ANOVA and Tukey, $p < 0.05$).

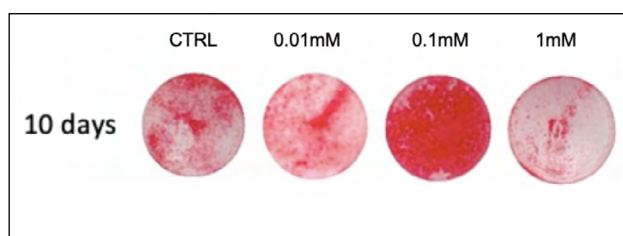


Fig. 5. Representative images of alkaline phosphatase activity for the different MLT doses.

groups at 24h, 48h and 72h ($p < 0.05$, Fig. 8). A very similar pattern was observed for OPN secretion ($p < 0.05$, Fig. 9).

DISCUSSION

This study investigated the role of MLT in bone metabolism using an *in vitro* experimental model based on osteoblast cultures for gene expression as well as secretion of key proteins involved in bone physiology, namely COL-I and OPN, over time^{17,10,12,24,25}. MLT has been shown to stimulate proliferation and differentiation of human osteoblasts *in vitro*, as well as the synthesis of COL-I and other proteins of the bone matrix (OPN, OC). Additionally, it has been demonstrated that MLT has the potential to reduce the time of osteoblast differentiation and proliferation^{9,23,24} as a result of the increased expression of bone sialoproteins and enhanced ALP, OPN and osteocalcin activity^{10,18}.

It has been demonstrated that the cytoprotective effects of MLT on osteoblasts are noted mainly at low concentrations and shorter times, using concentrations of 1, 10, 50, 100 and 200 mM for incubation periods of 24, 48 and 72h, showing the cytoprotective effect promoted by MLT in cells treated with bisphosphonate^{26,27,28,29}. There is, however, great variability in MLT concentrations across the literature, ranging from 0.001 to 2.5mM in osteoblastic cells of the MC3T3 lineage^{9,10,18}. In the present study, the higher concentrations used, 2.5mM and 5mM, proved cytotoxic, so they were excluded from the subsequent experiments. The 0.01, 0.1 and 1 mM concentrations were maintained. The time of 21 days is required to allow bone cells to proliferate, differentiate and mineralize *in vitro*, though considering the potential of melatonin to accelerate this process⁹, the times used in the present investigation were reduced to 10 days. MLT had a substantial effect on COL-I and OPN expression, including decreasing cell differentiation time to 5 - 7 days¹⁵, which has guided the design of the present study to evaluate phenotypical features at earlier stages of bone neoformation (10 days as opposed to 14 days). The findings presented herein therefore demonstrate that MLT enhances the process of osteogenesis. This may be attributed to an increase in the expression of bone sialoproteins and alkaline phosphatase activity, activated by the presence of MLT^{10,24}. The data obtained in the present study demonstrate that the metabolism of osteoblastic cells was positively affected by the MLT, mainly in relation to the calcium nodule count. On the 10th day, the alizarin red assay showed that all MLT concentrations were able to promote greater

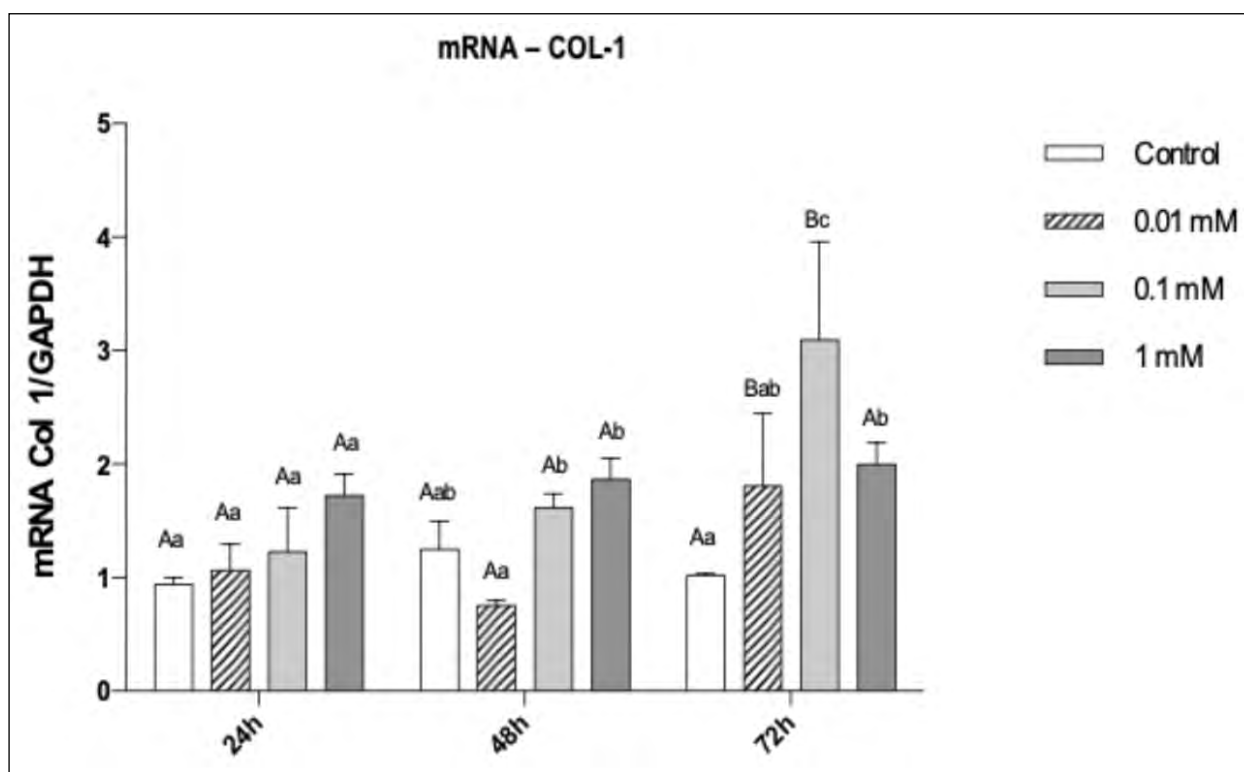


Fig. 6. mRNA expression for COL-1 in pre-osteoblastic cells as a function of MLT dose and time. Different uppercase letters represent a significant difference between the evaluation periods for each studied condition (ANOVA and Tukey, $p < 0.05$); different lowercase letters represent a significant difference between the concentrations tested (ANOVA and Tukey, $p < 0.05$).

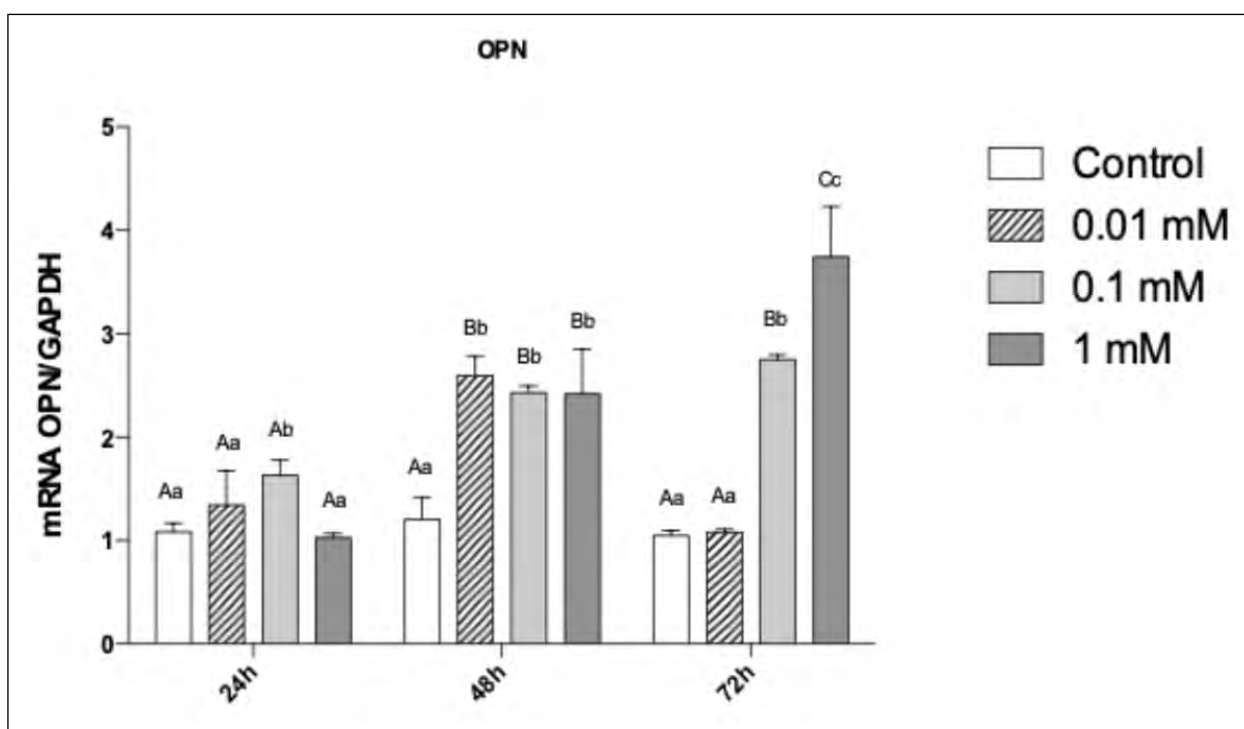


Fig. 7. mRNA expression for OPN in pre osteoblastic cells as a function of MLT dose and time. Different uppercase letters represent a significant difference between the evaluation periods for each studied condition (ANOVA and Tukey, $p < 0.05$); different lowercase letters represent a significant difference between the concentrations tested (ANOVA and Tukey, $p < 0.05$).

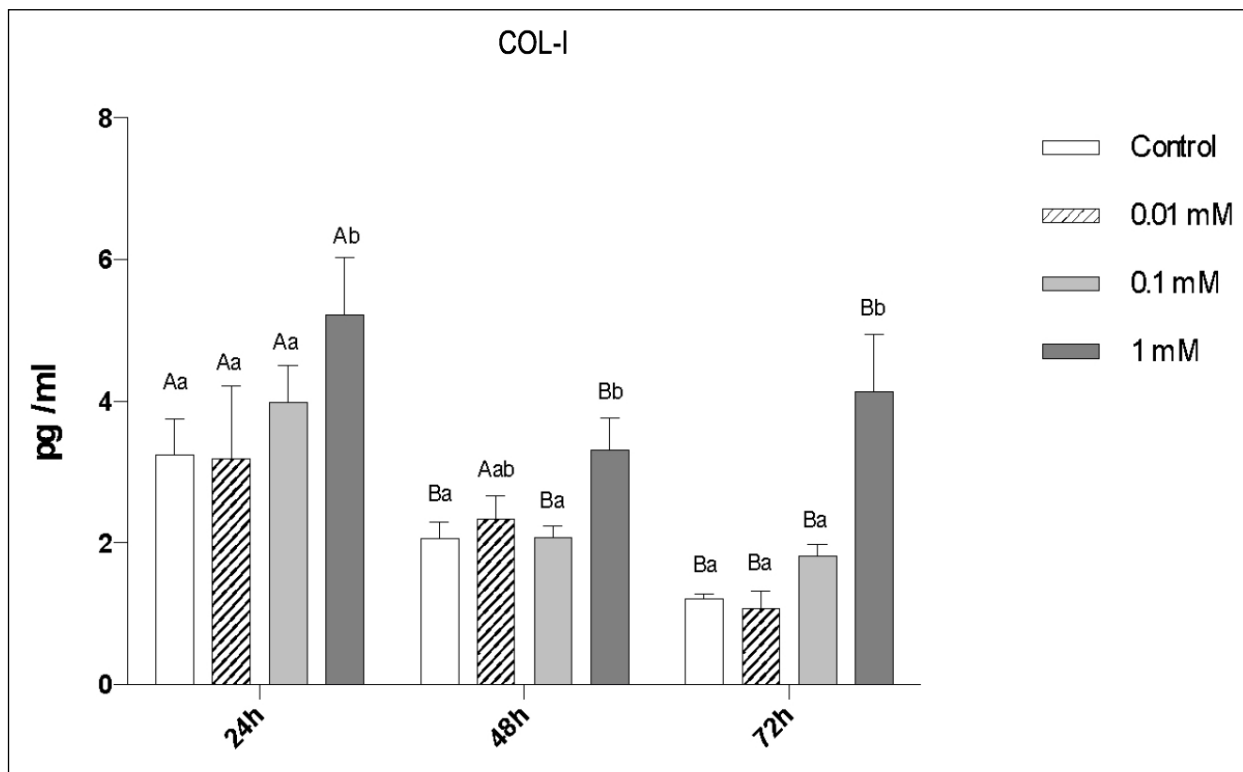


Fig. 8. ELISA for COL-I secretion by pre-osteoblastic cells as a function of MLT dose and time. Different uppercase letters represent a significant difference between the evaluation periods for each studied condition (ANOVA and Tukey, $p < 0.05$); different lowercase letters represent a significant difference between the concentrations tested (ANOVA and Tukey, $p < 0.05$).

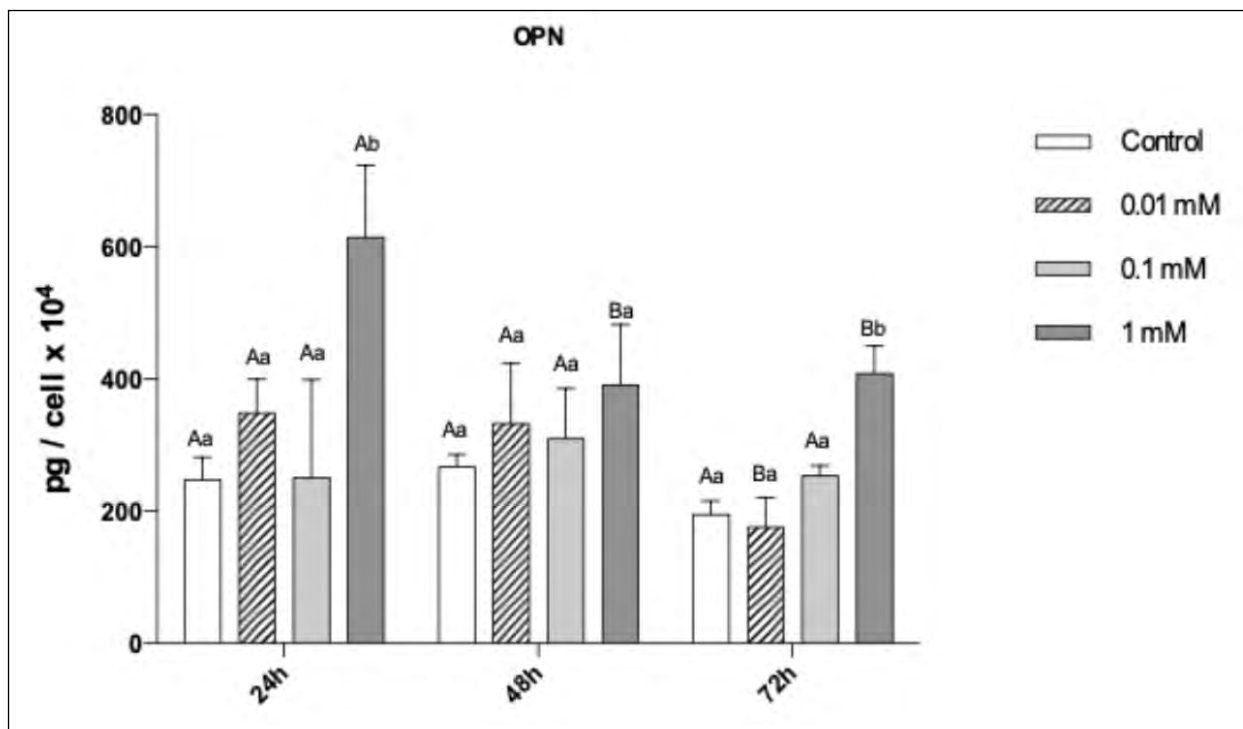


Fig. 9. ELISA for OPN secretion by pre-osteoblastic cells as a function of MLT dose and time. Different uppercase letters represent a significant difference between the evaluation periods for each studied condition (ANOVA and Tukey, $p < 0.05$); different lowercase letters represent a significant difference between the concentrations tested (ANOVA and Tukey, $p < 0.05$).

formation of mineral nodules than the control group, especially at the therapeutic dose of 0.1mM. Previous studies^{10,12,24} investigated the direct impact of MLT on pre-osteoblastic cells using increasing doses from 0.001mM to 2.5mM to determine its effects on metabolic activity, cell viability and gene expression of markers relating to osteoclastic activation. Subsequently, the best dose (0.1mM) was applied to assess mineralization, demonstrating that when evaluated using the alizarin red assay at 14 days, a greater formation of mineral nodules was observed in the group treated with MLT, in agreement with the results of the present study.

The alizarin red assay demonstrated the largest quantities of calcium nodules in the presence of 0.1mM MLT. The higher rate of mineralization in the group treated with melatonin at 0.1mM may indicate this as most beneficial in an *in vitro* context. In addition, higher doses can negatively interfere with cell mineralization, demonstrating that there is probably an ideal dose, in which the effect is enhanced, without being cytotoxic^{10,12,24}.

In the present study, alkaline phosphatase activity was verified at day 10 and an increase in mineral nodules was observed for the concentration of 0.1mM MLT, in agreement with studies that suggest that the hormone may have a regulatory effect on bone growth^{26,27}. Previous studies^{13,14,28} demonstrated that melatonin was able to regulate osteoblastic function via the MT2 receptor, which showed greater expression in bone during the experiments. In addition, inactivation of this receptor, leaving only MT1 receptor active, negatively affected cell differentiation, proliferation and deposition of the mineralized matrix, thus reiterating the promising role of melatonin as a potential strategy to stimulate bone neoformation.

In an *in vivo* study performed on a rodent experimental model of induced periodontitis¹⁹, treatment with MLT reduced serum COL-I

and increased ALP levels, demonstrating that expression of essential proteins occurred early in the cell differentiation process, as a result of MLT treatment. MLT has an anabolic effect on bone tissue, enhancing mineralization of MC3T3 cells¹². The MLT-treated cells showed a significant increase in COL-1 and OPN secretion²⁰⁻²². COL-1 and OPN have important roles in organic matrix formation and structural integrity, and are considered key to the mineralization of bone tissue^{10,18,19}. These findings were also observed herein for COL-I expression, which increased at 72h at 0.1 mM and 1mM of MLT, and for OPN expression at 1 mM, with concomitant increased ALP at 0.1 mM. All such findings are corroborated by studies performed elsewhere^{12,25}, though a considerable variation in therapeutic doses is observed across such studies.

The present study has demonstrated a potentially beneficial effect of MLT on MC3T3 cells in the early stages of osteogenesis, both in terms of cell proliferation and viability using doses of 0.01 mM and 0.1 mM, respectively, in addition to up-regulating the expression of COL-I and OPN, especially at 72h of treatment and doses of 0.1 mM and 1 mM, respectively. Increased secretion of COL-I and OPN followed suit at 1 mM and 24 h of treatment, thus corroborating previous studies on the regulatory effect of melatonin on bone growth^{10,13,15,21,22,24,25}. Meanwhile, several important issues remain to be addressed regarding the use of melatonin as a bone augmentation strategy. One of the most important is the lack of consensus regarding the most effective delivery mode, route of administration and dose^{30,25}. According to the findings in the present *in vitro* study, MLT at doses ranging from 0.01mM to 1 mM had a stimulatory effect on pre-osteoblasts by increasing the synthesis and secretion of both OPN and COL-I, as well as mineralization, thus favoring the process of osteogenesis.

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Effect of alternative solvent evaporation techniques on mechanical properties of primer-adhesive mixtures

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ABSTRACT

This study evaluated the influence of the mode and time of solvent evaporation on the tensile strength (TS), flexural strength (FS) and elastic modulus (EM) of two adhesive systems: Scotchbond Multipurpose (SBMP) and Clearfil SE (CSEB). For this purpose, rectangular samples (2x1x7 mm) were prepared with 10 µL of primer and the solvents were evaporated with air spray at (23±1) °C, (40±1) °C and negative control (without spray). For each temperature, the times of 5, 20, 30, and 60 seconds were investigated. The statistical results showed that evaporation at 40±1 °C resulted in better EM for the

two adhesives tested and all the evaporation times evaluated. However, there were no significant differences between the times and modes of evaporation for TS. The results of this study indicate that evaporation at a temperature of (40±1) °C could improve the elastic modulus of both adhesives tested, regardless of the evaporating time.

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Keywords: dentin bonding agents- tensile strength- flexural strength.

Efeito de técnicas alternativas utilizando evaporação de solvente nas propriedades mecânicas da mistura primer-adesivo

RESUMO

Este estudo avaliou a influência do modo e do tempo de evaporação do solvente na resistência à tração (RT), resistência à flexão (RF) e módulo de elasticidade (MR) de dois sistemas adesivos: Scotchbond Multipurpose (SBMP) e Clearfil SE (CSEB). Para isso, amostras retangulares (2x1x7 mm) foram preparadas com 10 µL de primer e os solventes foram evaporados com aerossol a (23±1) °C, (40±1) °C e controle negativo (sem aerossol). Para cada temperatura, foram avaliados os tempos de 5, 20, 30 e 60 segundos. Os resultados estatísticos mostraram que a evaporação a (40±1) °C resultou

em melhor MR para os dois adesivos testados e todos os tempos de evaporação avaliados. No entanto, não houve diferenças significativas entre os tempos e modos de evaporação na RT. Os resultados deste estudo indicam que a evaporação a uma temperatura de (40±1) °C poderia melhorar o módulo de elasticidade de ambos os adesivos testados, independentemente do tempo de evaporação.

Palavras-chave: adesivos dentinários- temperatura- resistência à tração- propriedades de flexão.

INTRODUCTION

Dentin hybridization through monomeric interdiffusion is an essential mechanism to obtain an effective, intimate bond to the dentin tissue¹. However, the bond between the dental substrate and the restorative

material is affected by the presence of residual water or excess solvent. The residual solvent in these systems may act as a polymeric matrix plasticizer, interfering with the mechanical properties of these materials and consequently with the bonding interface^{2,3}.

The solvents in the composition of adhesive systems should be completely evaporated from the resin-infiltrated dentin matrix^{4,6}. Excess water or other solvents from inadequate evaporation may negatively affect the physical-mechanical strength of the adhesion layer due to the inhibition of the polymerization of these materials and the plasticization of the adhesive structure by the presence of solvent molecules⁵⁻⁷.

The influence of excess solvent on the bond strength and mechanical properties of adhesive systems has been tested in several *in vitro* studies, which reported quick, standardized results establishing the behavior of materials in conditions similar to those in clinical practice³⁻⁷. However, these studies evaluated only the pure adhesive solutions, without primer addition. There is therefore a need to evaluate the primer/adhesive mixture for better comparison with what actually happens in clinical practice.

It has been shown that mixing the adhesive with the primer solution decreases the mechanical resistance of adhesive systems because the polymerization process is inhibited by compounds in the primer^{8,9}. It is therefore important to determine the effect of solvent evaporation on the mechanical properties of primer/adhesive mixtures.

Considering the adverse effect of excess solvent on the polymerization of adhesive systems⁹, the creation of alternative techniques for better evaporating these solvents can provide greater adhesive longevity. Thus, the aim of this study was to test the hypothesis that the evaporation of the solvent at high temperatures may improve the mechanical properties of Scotchbond Multipurpose PlusTM and Clearfil SE BondTM.

MATERIALS AND METHODS

This study investigated two commercial adhesives: a conventional three-step adhesive system -

Scotchbond Multipurpose PlusTM (SBMP-3M ESPE, St. Paul, MN, USA) and a two-step self-etching adhesive - Clearfil SE BondTM (CSEB-Kuraray Medical Inc., Okayama, Japan).

These adhesive systems were selected because they are both the gold standard of their classes and have repeatedly shown excellent results in clinical and laboratory studies¹⁰⁻¹². Table 1 presents the composition of the adhesive systems and Fig. 1 shows the preparation of specimens for each adhesive.

2.1 Sample preparation

Bar specimens (1 mm × 2 mm × 7 mm) were prepared in a rectangular silicone mold from the mixture of primer and adhesive of the two systems used. A total 360 specimens were obtained (n = 5 for modulus of elasticity and flexural strength and n = 10 for maximum tensile strength).

The solvent was evaporated by air spray at room temperature (23±1) °C and by heated air spray (40±1) °C using a device with pressure, airflow, and controlled power to provide airflow at a constant temperature. The temperature of the hot air at the spray outlet was approximately 100 °C. A standard distance of 5 cm was set between the air spray outlet and the mold surface to obtain a temperature of (40±1) °C in the sample drying process.

Initially, 10 µL of primer of each adhesive system investigated was inserted in the silicone mold, followed by the primer evaporation process according to the mode and time proposed in the experimental design. After that, 20 µL of adhesive were added and carefully mixed with the primer for 60 s to prevent the incorporation of bubbles (as far as possible). The primer/adhesive ratio by weight was 1:3, in accordance with the protocol established by Burchardt and Merz¹³. The samples were dried

Table 1. Composition of the bonding agents

Adhesive system	Manufacturer	Composition*
Scotchbond Multipurpose Etch-and-rinse (SBMP)	3M ESPE, St Paul, MN	Primer: HEMA, polyalkenoic acid polymer, water Adhesive: Bis-GMA, HEMA, tertiary amines, photoinitiator (CQ)
Clearfil SE BOND Self-etch (CSEB)	Kuraray, Osaka, Japan	Primer: MDP, HEMA, photoinitiator, water hydrophilic dimethacrylate Adhesive: MDP, HEMA, Bis-GMA, hydrophobic dimethacrylate, photoinitiators, colloidal silica

Abbreviations - BisGMA: bisphenol-A glycidyl dimethacrylate; HEMA: 2-hydroxyethylmethacrylate; CQ: camphorquinone; MDP: 10-methacryloyloxydecyl dihydrogen phosphate *As informed by the manufacturers.

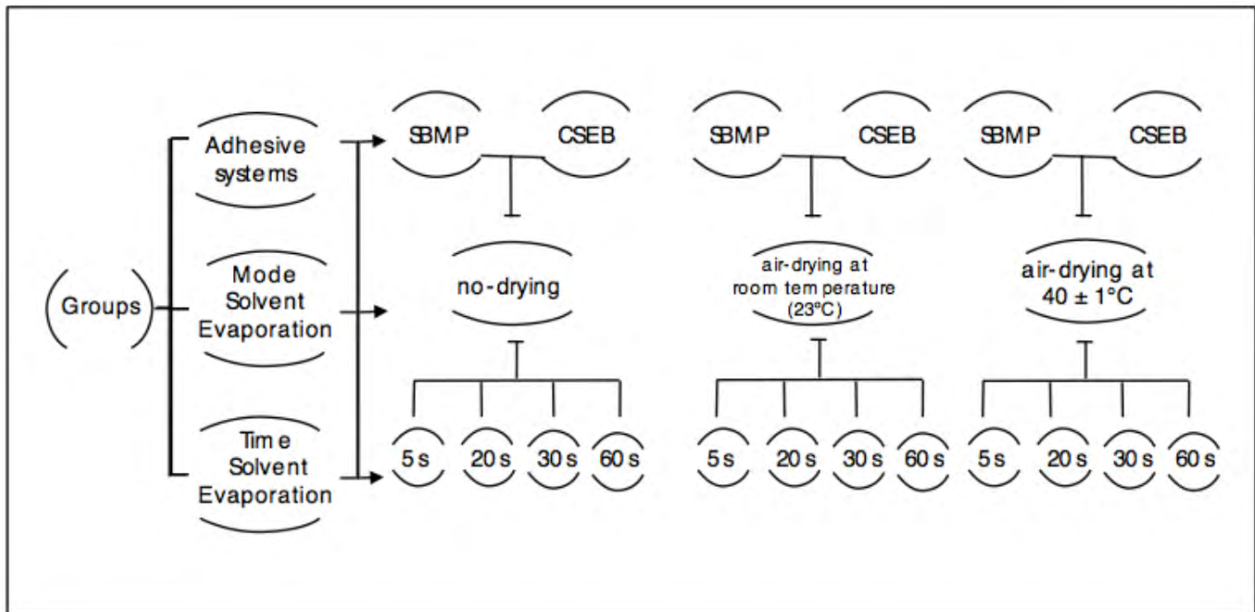


Fig. 1. Experimental design.

at different temperatures and photopolymerized using the Vip Bisco (Vip Curing Lights - BISCO Dental Products IL, USA) with 600 mw/cm² of light intensity.

The light was activated with a holder coupled to the light sources to standardize a 4 mm distance between the light guide tip and the material, which simulated a medium cavity depth. The polymerized samples were removed from the silicon mold and stored in distilled water for 24 h at 37 °C.

2.2 Measurement of tensile strength (TS)

The dimensions of the specimens were measured with a digital caliper (Digimatic Caliper, Mitutoyo, Tokyo, Japan). Bar-shaped specimens were attached to a device with cyanoacrylate adhesive and tested under tension in a Universal Testing Machine (EMIC Ltda., São José dos Pinhais, SP, Brazil) at 0.5 mm/min speed until rupture. The TBS values were calculated in MPa by dividing the load at rupture by the cross-sectional area.

2.3 Measurement of flexural strength (FS) and elastic modulus (EM)

The specimens were subjected to a three-point bending flexural test to measure flexural strength (FS) and elastic modulus (EM). The three-point bending test was performed in a universal testing machine (Instron model 4411, Instron Corp., Canton, MA, USA) according to ISO 4049 specifications.

Before the test, the dimensions of each specimen were recorded with a caliper. The Bluehill 2 software (Instron Corp., Canton, MA, USA) was used to calculate EM (GPa) and FS (MPa) values according to the dimensions and tension in each case. The maximum loads were obtained and the flexural strength (σ) was calculated in megapascals (MPa) by using the formula:

$$\sigma = 3FL/(2BH^2)$$

where F is the maximum load (in newtons); L is the distance between the supports (in millimeters); B is the width of the specimen (in millimeters) and H is the height (also in millimeters).

The modulus of elasticity (GPa) was calculated as:

$$E = FL^3/4BH^3d$$

where F is the maximum load; L is the distance between the supports; B is the width of the specimen, H is the height of the specimen, and d is the deflection (in millimeters) corresponding to the load F.

2.4 Statistical analysis

The data were analyzed with one-way ANOVA and Tukey's post-hoc test at a 99.5% confidence level. In addition, they were subjected to multivariate analysis of variance (MANOVA) with repeated measures and a Lambda Wilks test ($p < 0.05$).

RESULTS

Based on the elastic modulus results (Table 2), the evaporation at $\pm 40^\circ\text{C}$ resulted in a higher elasticity modulus, regardless of the time of application of the air spray and the adhesive system used. The use of the heated air spray led to 1.59 GPa mean modulus value, which was statistically higher than the value found for the groups where spray at room temperature (1.40 GPa) or evaporation without application of air spray (1.37 GPa) were used.

As for the influence of time on the elastic modulus, the increase of the evaporation time did not affect the behavior of the adhesive systems investigated. There was an exception for the time of 5 s for the CSEB self-etching adhesive system, which showed a significant reduction in the elasticity modulus when compared to the time of 30 s for the same group (1.60 GPa) and 60 s for the conventional

adhesive system (1.56 GPa). The specimens from the other groups presented EM with statistically similar results (Table 3).

Flexural strength analysis showed that the change in the solvent evaporation regime did not lead to any significant results. However, comparing the systems evaluated, the CSEB self-etching adhesive system (84.04 MPa) was found to have better properties than the SBMP etch-and-rinse system (62.44 MPa) (Table 4).

The interaction between adhesive system, mode, and evaporation time did not present statistical difference concerning maximum tensile strength (Table 5).

DISCUSSION

The solvent present in adhesive systems acts as a transport vehicle by decreasing the viscosity

Table 2. Elastic modulus (EM) of the different solvent evaporation types

Air Spray	Evaporation times	Adhesive system	M/SD (GPa)	M/SD (GPa)*
No drying (ND)	5	SBMP	1.33 (0.09)	1.37 (1.02) B
		CSEB	1.10 (1.01)	
	20	SBMP	1.26 (1.00)	
		CSEB	1.30 (0.06)	
	30	SBMP	1.40 (0.08)	
		CSEB	1.60 (0.08)	
	60	SBMP	1.52 (1.02)	
		CSEB	1.41 (1.02)	
Air-drying at room temperature 23° C (AD23)	5	SBMP	1.35 (1.02)	1.40 (0.09) B
		CSEB	1.41 (0.09)	
	20	SBMP	1.36 (1.02)	
		CSEB	1.40 (1.01)	
	30	SBMP	1.36 (0.08)	
		CSEB	1.39 (1.00)	
	60	SBMP	1.56 (0.08)	
		CSEB	1.37 (0.06)	
Air-drying at 40°C $\pm$ 1°C (AD40)	5	SBMP	1.52 (0.09)	1.59 (1.32) A
		CSEB	1.62 (1.31)	
	20	SBMP	1.61 (1.20)	
		CSEB	1.52 (0.16)	
	30	SBMP	1.60 (0.18)	
		CSEB	1.66 (0.68)	
	60	SBMP	1.60 (1.32)	
		CSEB	1.59 (1.32)	

*M: Mean; SD: Standard deviation; Means followed by different letters differ from each other ($p \leq 0.05$).

Table 3. Elastic modulus (EM) of the different solvent evaporation times

Evaporation times	Air Spray	Adhesive system	M/SD (GPa)	M/SD (GPa)*
5	No drying (ND)	SBMP	1.33 (0.09)	1.46 (0.09) A
		CSEB	1.10 (1.01)	
	Air-drying at room temperature 23° C (AD23)	SBMP	1.35 (1.02)	
		CSEB	1.41 (0.09)	
	Air-drying at 40°C ± 1°C (AD40)	SBMP	1.52 (0.09)	
		CSEB	1.62 (1.31)	
20	No drying (ND)	SBMP	1.26 (1.00)	1.41 (1.01) A
		CSEB	1.30 (0.06)	
	Air-drying at room temperature 23° C (AD23)	SBMP	1.36 (1.02)	
		CSEB	1.40 (1.01)	
	Air-drying at 40°C ± 1°C (AD40)	SBMP	1.61 (1.20)	
		CSEB	1.52 (0.16)	
30	No drying (ND)	SBMP	1.40 (0.08)	1.46 (1.00) A
		CSEB	1.60 (0.08)	
	Air-drying at room temperature 23° C (AD23)	SBMP	1.36 (0.08)	
		CSEB	1.39 (1.00)	
	Air-drying at 40°C ± 1°C (AD40)	SBMP	1.60 (0.18)	
		CSEB	1.66 (0.68)	
60	No drying (ND)	SBMP	1.52 (1.02)	1.48 (1.02) A
		CSEB	1.41 (1.02)	
	Air-drying at room temperature 23° C (AD23)	SBMP	1.56 (0.08)	
		CSEB	1.37 (0.06)	
	Air-drying at 40°C ± 1°C (AD40)	SBMP	1.60 (1.32)	
		CSEB	1.59 (1.32)	

*M: Mean; SD: Standard deviation; Means followed by different letters differ from each other (p≤0.05).

of the adhesive, thereby enabling the resinous monomers to penetrate the micropores formed by acid etching^{13,14}. However, incomplete solvent evaporation significantly compromises adhesion efficacy^{3-7,14}, considering that excess solvents can lead to a dilution of the components that prevent the collision of reactive components. This hinders the attainment of high cross-linking polymers inside the hybrid layer, which may result in a porous hybrid layer structure with less mechanical properties¹⁵. The results showed that both drying time and temperature had a significant effect on the mechanical properties of the two adhesive systems tested. The application of hot air spray (40 ± 1°C) produced better elasticity modulus values, regardless of the adhesive system used. The elastic modulus represents the relative stiffness of the material as well as its resistance to plastic deformation, which is directly related to the formation of more crosslinks in

the polymer structure.² The increase in the modulus as a result of the heated air spray is probably due to the efficient removal of the solvent, leading to a more efficient polymerization process^{3,4,14}.

This phenomenon may be also explained based on the formulation of the two adhesive systems investigated in this study, in which the primer formulation contains HEMA and water. The presence of HEMA is important because it can expand the dried dentin collagen and improve adhesion. However, the presence of HEMA in these systems improves the retention of solvent and water in polymer networks due to its influence on resin polarity. The presence of HEMA provides the high number of OH groups in the polymeric chains and improves the hydrogen bonding sites between polymer and solvent, hindering solvent evaporation¹⁶. In this case, the use of temperature for solvent elimination is important to better evaporate the water in the system.

Table 4. Flexural strength (FS) according to adhesive system interaction, time and type of solvent evaporation

Air Spray	Evaporation times	Adhesive system	M/SD (MPa)	M/SD (MPa)*
No drying (ND)	5	SBMP	60.55 (22.09)	72.52 (35,71) A
		CSEB	84.42 (18.09)	
	20	SBMP	60.11 (19.04)	
		CSEB	80.40 (19.99)	
	30	SBMP	64.57 (31.05)	
		CSEB	81.01 (22.09)	
	60	SBMP	63.01 (22.09)	
		CSEB	86.04 (21.01)	
Air-drying at room temperature 23° C (AD23)	5	SBMP	62.09 (18.09)	73.77 (22.50) A
		CSEB	88.04 (21.01)	
	20	SBMP	64.21 (25.87)	
		CSEB	83.00 (21.01)	
	30	SBMP	65.31 (24.00)	
		CSEB	82.81 (21.01)	
	60	SBMP	58.65 (28.01)	
		CSEB	86.04 (21.01)	
Air-drying at 40°C ± 1°C (AD40)	5	SBMP	59.88 (19.09)	73.44 (20.16) A
		CSEB	88.35 (21.07)	
	20	SBMP	65.77 (20.34)	
		CSEB	79.04 (18.00)	
	30	SBMP	64.63 (19.53)	
		CSEB	85.24 (20.01)	
	60	SBMP	60.50 (22.23)	
		CSEB	84.09 (21.01)	

*M: Mean; SD: Standard deviation; Means followed by different letters differ from each other ($p \leq 0.05$).

In these adhesives with HEMA/water mixtures, the water evaporates more rapidly than the HEMA, which is a relatively non-volatile component. Thus, the concentration of HEMA increases as the water evaporates, reducing the vapor pressure of the water and making the adhesive more viscous, which makes it difficult to remove the last remaining water^{16,17}.

The difference in the properties of the two adhesive systems compared in this study may be due to their composition. The primer in the self-etching adhesive system (CSEB) contains acid monomers, HEMA, and water, while the primer in the conventional three-step adhesive system (SBMP) contains only water and HEMA. The incorporation of acid monomers in the simplified adhesive systems is necessary to condition the dental structures. However, such monomers may increase the water absorption by these systems due to the interaction of monomers and water¹⁸.

The interaction between adhesive and time shows that the increase of water concentration results in an adhesive interface with loss of mechanical properties, higher water absorption, and a lower degree of conversion^{18,19}. The increase in water concentration results in increased ionization of acid monomers, which may reduce their reactivity and the polymerization of the systems^{18,20}. Thus, the lowest mechanical property exhibited at 5 s for all adhesives may be due to the high concentration of residual water that did not allow adequate polymerization of these monomers.

Tensile strength evaluates the cohesion in the polymer layer formed after polymerization. Thus, increasing cohesion indicates a formation of a hybrid layer with lower microporosity, which is consequently less susceptible to infiltration². In this study, there was no statistical difference in tensile strength, indicating that the evaporation obtained was not

Table 5. Maximum tensile strength (TS) according to adhesive system interaction, time and type of solvent evaporation

Air Spray	Evaporation times	Adhesive system	M/SD (MPa)	M/SD (MPa)*
No drying (ND)	5	SBMP	90.05 (5.81)	93.84 (8.05) A
		CSEB	95.34 (9.77)	
	20	SBMP	95.32 (9.76)	
		CSEB	91.11 (8.96)	
	30	SBMP	94.08 (7.87)	
		CSEB	95.14 (6.65)	
	60	SBMP	94.45 (9.83)	
		CSEB	95.23 (5.75)	
Air-drying at room temperature 23° C (AD23)	5	SBMP	91.25 (6.72)	94.33 (6.21) A
		CSEB	96.44 (9.09)	
	20	SBMP	95.42 (4.67)	
		CSEB	92.23 (8.93)	
	30	SBMP	94.18 (5.45)	
		CSEB	95.24 (6.33)	
	60	SBMP	94.55 (3.26)	
		CSEB	95.33 (5.23)	
Air-drying at 40°C ± 1°C (AD40)	5	SBMP	99.33 (9.01)	94.10 (8.93) A
		CSEB	94.06 (9.34)	
	20	SBMP	94.19 (9.20)	
		CSEB	90.32 (8.08)	
	30	SBMP	93.14 (9.06)	
		CSEB	94.08 (9.32)	
	60	SBMP	93.23 (8.09)	
		CSEB	94.45 (9.34)	

*M: Mean; SD: Standard deviation; Means followed by different letters differ from each other (p≤0.05).

sufficient to improve material cohesion. The result does not corroborate previous studies that show the increase of cohesive resistance against the change in solvent evaporation^{3-7,14}. However, those studies did not use the primer/adhesive mixture, which may be responsible for the difference observed.

Similarly, the evaluation of flexural strength showed no statistical difference regarding the different times and modes of solvent evaporation. For flexural strength, it was observed that the CSEB adhesive system obtained better results than the SBMP. The evaluation of this property is important for clinical practice, considering that the adhesive layer must undergo the least flexing when submitted to masticatory loads and this mechanical property can evaluate the resistance of the material to deformity as well as the tensile strength².

As mentioned above, the adhesive systems used have

different primer compositions. The CBSE contains, along with HEMA and water, acidic monomers that are hydrophilic and favor higher water adsorption by the primer of the adhesive system, making it less viscous. The lower the viscosity of a fluid, the faster and more easily it will move and thus allow greater interaction among the monomers, resulting in a more complete polymerization reaction with a better quality of crosslinks and providing greater cohesion to the polymers¹⁴.

According to the results of this study, evaporation time did not provide a positive correlation between TBS and EM. The EM of the adhesives tested was increased with hot air evaporation, while flexural strength did not change. It is therefore believed that the evaporation of the solvent with heated air spray provides a greater number of crosslinks, but a better quality between these bonds could not be obtained.

CONCLUSIONS

The results of the present study show that:

- The different solvent evaporation modes and temperatures did not affect the flexural strength of the adhesives tested.
- The CSEB system obtained better results than

CBMP regarding flexural strength.

- Hot air spray evaporation (40°C) obtained better results of elasticity modulus (relative rigidity of the material), i.e., a greater number of crosslinks regardless of the adhesive system and time of evaporation.

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Signs of alveolar bone damage in early stages of periodontitis and its prevention by stimulation of cannabinoid receptor 2. Model in rats

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ABSTRACT

The aims of the present study were, first, to identify signs of alveolar bone damage in early stages of experimental periodontitis (EP) and, second, to assess its possible prevention by treatment with cannabinoid receptor 2 agonist HU 308. Experimental periodontitis was induced by injections of lipopolysaccharide (LPS) (1mg/ml) in gums surrounding maxillary and mandibular first molar, 3 days per week, and untreated controls were kept for comparison. Then, a 3-week study was conducted including eighteen new rats (six rats per group): 1) controls; 2) experimental periodontitis rats; and 3) experimental periodontitis rats treated daily with HU 308 (500 ng/ml). After euthanasia, alveolar bone loss was assessed by morphometric and histomorphometric techniques, and the content of prostaglandin E₂ (PGE₂) in gingival tissue was evaluated by radioimmunoassay. The first signs of alveolar bone loss were apparent at 3 weeks of experimental periodontitis ($p < 0.05$) in the mandibular first molar, but there was no

detectable change at 1 week, leading us to establish 3 weeks as an early stage of experimental periodontitis. Rats subjected to 3-week experimental periodontitis showed less interradicular bone volume, less whole bone perimeter and fewer bone formation areas, and higher periodontal space height, bone resorption areas, number of osteoclasts and gingival content of prostaglandin E₂ than controls, while HU 308 prevented, at least partially, the deleterious effects ($p < 0.001$). We can conclude that a 3-week term of lipopolysaccharide-induced periodontitis in rats provides a valid model of the early stage of the disease, as emerging damage is observed in bone tissue. Furthermore, harmful effects at 3 weeks could be prevented by local stimulation of cannabinoid receptor 2, before greater damage is produced.

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Keywords: periodontitis- cannabinoids- alveolar bone loss- prostaglandin E₂.

Signos de daño óseo alveolar en estadios tempranos de periodontitis y su prevención por estimulación del receptor cannabinoide 2. Modelo en ratas microcizalhamento do cimento resinoso

RESUMEN

El objetivo del presente trabajo fue, en primer lugar, identificar signos de daño óseo alveolar en estadios tempranos de periodontitis experimental y, en segundo lugar, evaluar su posible prevención mediante el tratamiento con el agonista del receptor cannabinoide 2, HU 308. La periodontitis experimental fue inducida por inyecciones de lipopolisacárido (1mg/ml) en la encía circundante al primer molar maxilar y mandibular; 3 días por semana, en tanto que controles no tratados fueron mantenidos para la comparación. Posteriormente, un estudio de 3 semanas con dieciocho nuevas ratas (seis por grupo) fue desarrollado: 1) controles; 2) ratas con periodontitis experimental, y 3) ratas con periodontitis experimental tratadas diariamente con HU 308 (500ng/ml). Luego de la eutanasia, la pérdida ósea alveolar fue evaluada por técnicas morfométricas e histomorfométricas, y el contenido de prostaglandina E₂ en el tejido gingival fue determinado por radioinmunoensayo. Los primeros signos de pérdida ósea alveolar fueron evidentes a las 3 semanas de inducción de periodontitis experimental ($p < 0.05$) en el primer molar mandibular; mientras

que no hubo cambios detectables luego de 1 semana de inducción, hecho que nos condujo a establecer a las 3 semanas como un estadio temprano de periodontitis experimental. Las ratas sometidas a periodontitis experimental de 3 semanas mostraron menor volumen óseo interradicular; menor perímetro óseo y menos áreas de formación ósea, y mayor altura del espacio periodontal, más áreas de reabsorción ósea, mayor número de osteoclastos y mayor contenido gingival de prostaglandina E₂, en comparación a los controles, mientras que el tratamiento con HU 308 previno, al menos parcialmente, los efectos deletéreos ($p < 0.001$). Podemos concluir que el término de 3 semanas de periodontitis inducida por lipopolisacárido es un modelo válido de estadio inicial de la enfermedad experimental, dado que se evidencia daño emergente en el tejido óseo. Asimismo, los efectos deletéreos de 3 semanas podrían ser prevenidos por la estimulación local del receptor cannabinoide 2, antes que un daño mayor sea producido.

Palabras clave: periodontitis, cannabinoides, pérdida ósea alveolar; prostaglandina E₂.

INTRODUCTION

Initial stages of periodontal diseases include gingivitis and incipient chronic periodontitis. Gingivitis is defined as an inflammation of the gingival tissue, while periodontitis involves the loss of attachment of periodontal tissues from the tooth and the loss of alveolar bone. Gingivitis is reversible, whereas regeneration after the destruction caused by periodontitis is not predictably achievable in humans¹. Even though most periodontal diseases are known to occur in susceptible subjects, their onset also depends on colonization by bacteria and presence of bacterial products². Lipopolysaccharide (LPS), a toxin produced by gram-negative microorganisms, can cause an inflammatory process, with the consequent damage in the tooth-insertion tissues³. In the host tissues, LPS stimulates phagocytes to increase the production of cytokines such as TNF α , IL-1 β , IL-6, IL-10, IL-12 and IL-15⁴. Those mediators are responsible for a large number of cellular events, including the synthesis of PGE₂⁵, a potent stimulator of bone resorption associated with loss of periodontal attachment tissues⁶. Additionally, in animals with periodontitis, PGE₂ has been found to increase in the submandibular gland, where it seems to function as an inhibitor of salivary secretion⁷. Thus, although colonization by pathogenic microbes is the primary origin of damage in periodontal diseases, the host immune-inflammatory response plays a critical role in the development of tissue injury resulting in the loss of connective tissue and alveolar bone⁸.

The cellular and humoral response deployed in bacterial processes involves the endocannabinoid system (ECS)^{9,10}, which is an intercellular communication network that includes receptors, endogenous ligands and a series of enzymes responsible for ligand synthesis and breakdown. Cannabinoid receptors type 1 (CB1r) and type 2 (CB2r) are the main specific cannabinoid receptors, whereas transient receptor potential vanilloid type 1 is the most thoroughly studied unspecific receptor¹¹. According to the classical concept and even though it has been submitted to revisions, CB1 is highly expressed in the central nervous system and to a lesser extent, in peripheral tissues. Conversely, CB2 is expressed mainly in peripheral tissues and cells such as monocytes, macrophages, lymphocytes and bone cells¹². However, the stimulation of ECS has been associated with anti-inflammatory

effects through both CB1^{13,14} and CB2¹⁵ receptors. In previous studies, we have shown that in a six-week LPS-induced periodontitis model in rats, daily application of a CB2r synthetic agonist, HU 308, attenuated alveolar bone loss and reduced the level of inflammatory mediators in gums¹⁶.

The aims of this study were, firstly, to identify early alveolar bone damage in periodontal disease in order to establish initial stages of the disease in an experimental model, and secondly, to evaluate the effects of the treatment with a CB2 receptor agonist, HU 308, on the oral health of rats submitted to early periodontitis, and compare these effects with those found in the pattern of long-term illness.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (350 g) from the authors' own colony were kept in group cages in an animal room with a 12-hour light photoperiod (0700 to 1900), room temperature maintained at 22°C to 25°C, and free access to rat chow and tap water. The experimental procedures performed were approved by the Animal Care Committee of the Dental School of the University of Buenos Aires, Buenos Aires, Argentina and carried out in accordance with guidelines of the National Institutes of Health.

Time range studies

Since our previous studies had been based on a six-week lipopolysaccharide-induced periodontitis model, we intended to achieve a model of periodontitis with emerging damage. To this end, we conducted a first experiment based on temporal studies of lipopolysaccharide-periodontitis induction at 1, 3 and 6 weeks. For each term of induction, twelve rats were divided into two groups, each group containing six rats: 1) control rats, and 2) rats submitted to experimental periodontitis. Periodontitis was induced by injecting 20 μ l of lipopolysaccharide (1 mg/ml) from *Escherichia coli* into the vestibular and lingual gingiva of the maxillary and mandibular first molars and into the interdental space between the first and second maxillary and mandibular molars (60 μ l of lipopolysaccharide per tooth and 240 μ l per rat at each time of injection). Gingival injections were all performed using a 13-mm 27-gauge microfine insulin syringe. Control rats received no injection during the experiments.

CB2 receptor studies

In a second experiment, eighteen rats were distributed into three groups of six rats per group: 1) control rats; 2) rats submitted to EP; and 3) rats submitted to EP and treated with HU 308, an agonist of CB2r. Periodontitis was induced as described for the preliminary studies, while control rats received no injection during the experiment. This protocol of injections was executed for a period of 3 weeks on days 1, 3, and 5 of each week, based on a previously described method^{14,17}. Gingival injections were applied using a 13-mm 27-gauge microfine insulin syringe.

Topical treatment with a CB2 receptor agonist

HU 308 ([1R,2R,5R)-2-[2,6-dimethoxy-4-(2-methyloctan-2-yl) phenyl]-7,7-dimethyl-4-bicyclo [3.1.1] hept-3-enyl] methanol) was prepared by dissolving the powdered drug in 100% ethanol and diluting it in saline, containing approximately 1% ethanol, to obtain a final concentration of 500 ng/ml with which to treat animals.

The volume of each topical application was 200 µl per tooth, resulting in 800 µl per animal when treatment was performed. As mentioned above, the second experiment consisted of a 3-week study in which group 3 animals received a daily topical application of HU 308 at sites of LPS during the 3 weeks of the experiment. The optimal dose of HU 308 was based on previous reports using anandamide, but mainly using methanandamide, a selective synthetic agonist of CB1 receptor^{14,18}, on oral tissues in vivo concomitantly with the dose response curves in these preliminary studies.

Macroscopic examination of periodontal bone loss: distance and width methods

Immediately after euthanasia of the rats, hemimandibles were resected, defleshed, and stained with 1% aqueous methylene blue to delineate the cementoenamel junction (CEJ) and the alveolar crest (AC)¹⁹. A stereomicroscope and a digital calliper were used to measure three buccal and three lingual/palatal distances (mesial, central, and distal), from the CEJ to the AC¹⁴. The sum of the three distances on each side of molars was used as a measure of alveolar bone loss (ABL) in millimetres. Thus, ABL was assessed on the buccal side, palatal/lingual side and also as whole tooth of the first upper and lower molars. Mandibular alveolar process

width was measured in the mandibular first molar area. Distance between a point located at the central root level of the buccal surface and another equally located on the lingual surface was measured in the mandibles in millimetres using a digital calliper. This examination was performed for all the groups in the first and second experiments.

Histological analysis

Hemimandibles on the opposite side to those used for macroscopic examinations were fixed in formalin buffer. After 3 days, they were decalcified in 10% EDTA pH 7 for 45 days. Then hemimandibles were dehydrated with ethyl alcohol and clarified with xylene. Finally, the sector containing the first molar of each decalcified hemimandible was embedded in paraffin at 56°C to 58°C. Under the stereomicroscope, sections oriented mesial-distally of each mandibular first molar were cut from the paraffin blocks using a microtome. Sections 5 µm thick were stained with haematoxylin and eosin (H&E), and histomorphometric evaluation was performed on digitized microphotographs using imaging software. Interradicular bone loss was evaluated by measuring the periodontal space height, plotting 10 equidistant lines between the alveolar crest and the cementum of the furcation zone. The length of the lines was measured and the mean value was calculated. Additionally, ABL was assessed by the following parameter: bone volume (B.Ar)/total volume (T.Ar) (%) = fraction of TV corresponding to bone tissue. T.Ar was taken as bone tissue plus bone marrow and periodontal ligament²⁰. The remaining histomorphometric parameters were evaluated in the bone of the interdental septum located between the first and second lower molars. These parameters were: whole perimeter of the interdental septum, in µm; bone formation areas, in % as Ob.S/BS (osteoblast surface related to total bone surface); bone resorption areas, in % as ES/BS (eroded surface related to total bone surface), and number of osteoclasts^{21,22}, as N.Oc/mm, in the cervical third of the mentioned septum. Histological analysis was performed for all the groups in the second experiment.

Radioimmunoassay of PGE₂

To determine PGE₂ content, gingival tissue surrounding first molar was homogenized in 500 ml absolute ethanol and after centrifugation,

supernatants were dried in a centrifugal vacuum concentrator at room temperature. Residues were then resuspended with buffer, and antiserum was used as described in Mohn et al.²³. Sensitivity of the assay was 12.5 pg per tube. The cross-reactivity of PGE₂ and PGE₁ was 100%, but the cross-reactivity of other prostaglandins was 0.1%. The intra- and inter-assay coefficients of variation for PGE₂ were 8.2% and 12.0%, respectively. The results were expressed in picograms of PGE per milligram of wet weight, since the protocol of PGE extraction from the tissue includes homogenization in ethanol, which interferes with protein determination. This evaluation was performed for all the groups in the first and second experiments.

Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean. Results were evaluated by one-way analysis of variance followed by the Tukey multiple comparisons test for unequal replicates. Only the number of osteoclasts was analysed using the Kruskal-Wallis nonparametric test. All analyses were conducted with Prism software and differences with P values <0.05 were considered statistically significant.

RESULTS

Time range studies

• Alveolar bone loss: distance and width methods

After 1 week of LPS injections, ABL measured by the distance method did not differ between control and LPS groups in either upper or lower first molars (Table 1). However, a significant alveolar bone loss

was noticeable after 3 weeks of LPS induction, especially when whole teeth were assessed (Table 2). Furthermore, the differences described were stronger after 6 weeks of LPS induction, when both sides of upper and lower first molars as well as the whole tooth showed an increase in alveolar bone loss in LPS compared to control group (Table 3). ABL measured by the width method in the mandible did not reveal changes between control and EP group after 1 and 3 weeks of procedure. In contrast, the width of the lower alveolar process was significantly lower in the LPS than in control rats after 6 weeks of induction (Table 3).

• Radioimmunoassay of prostaglandin E₂

PGE₂ content was evaluated as a marker of inflammatory response in gingival tissue surrounding the first molar. PGE₂ content measured by RIA was markedly higher in gingival tissue after 1, 3 and 6 weeks of LPS injections in comparison to the control group. Results for 1, 3 and 6 weeks are shown at the bottom of Tables 1, 2 and 3, respectively.

CB2 receptor stimulation in early stage-experimental periodontitis rats - Three-week studies

• Alveolar bone loss: distance and width methods

In early stage-experimental periodontitis rats, alveolar bone loss measured through the distance method did not show major changes among groups in the first upper molar when buccal and palatal sides were analysed separately. In contrast, the early-stage experimental periodontitis group experienced higher alveolar bone loss than control group at the lingual side of the first lower molar,

Table 1. Macroscopic evaluation of alveolar bone loss through distance and width methods, and gingival PGE₂ content measurement after one week of LPS applications.

Macroscopic ABL (mm)			Control	LPS
Distance	First upper molar	Buccal	2.38 ± 0.60	2.54 ± 0.56
		Palatal	2.83 ± 0.78	2.80 ± 0.53
		Whole tooth	5.21 ± 1.15	5.33 ± 1.03
	First lower molar	Buccal	2.45 ± 0.19	2.57 ± 0.45
		Lingual	3.56 ± 0.34	3.70 ± 0.50
		Whole tooth	6.01 ± 0.46	6.27 ± 0.58
Width	First lower molar		2.62 ± 0.04	2.53 ± 0.02
Gingival PGE ₂ (pg/mg tissue)			87.30 ± 12.99	154.79 ± 21.74*

* $p < 0.05$ vs. control. ABL: alveolar bone loss. PGE₂: prostaglandin E₂. LPS: lipopolysaccharide.

Table 2. Macroscopic evaluation of alveolar bone loss through distance and width methods, and gingival PGE₂ content measurement after three weeks of LPS applications.

Macroscopic ABL (mm)			Control	LPS
Distance	First upper molar	Buccal	2.70 ± 0.39	3.38 ± 0.64
		Palatal	3.00 ± 0.45	3.57 ± 0.71
		Whole tooth	5.70 ± 0.60	6.96 ± 1.12 *
	First lower molar	Buccal	2.75 ± 0.36	3.75 ± 0.37 *
		Lingual	4.37 ± 0.39	4.84 ± 0.39 *
		Whole tooth	7.12 ± 0.53	8.58 ± 0.62 *
Width	First lower molar	2.65 ± 0.03	2.53 ± 0.06	
Gingival PGE ₂ (pg/mg tissue)			58.97 ± 6.29	124.3 ± 26.13 *
*p<0.05 vs. control. ABL: alveolar bone loss. PGE ₂ : prostaglandin E ₂ . LPS: lipopolysaccharide				

*p<0.05 vs. control. ABL: alveolar bone loss. PGE₂: prostaglandin E₂. LPS: lipopolysaccharide

Table 3. Macroscopic evaluation of alveolar bone loss through distance and width methods, and gingival PGE₂ content measurement after six weeks of LPS applications.

Macroscopic ABL (mm)			Control	LPS
Distance	First upper molar	Buccal	2.78 ± 0.63	4.25 ± 0.65 **
		Palatal	3.26 ± 0.27	4.30 ± 0.24 ***
		Whole tooth	6.04 ± 0.28	8.56 ± 0.39 ***
	First lower molar	Buccal	2.83 ± 0.47	3.85 ± 0.71 *
		Lingual	4.08 ± 0.32	5.72 ± 0.58 ***
		Whole tooth	7.09 ± 0.30	9.30 ± 0.26 ***
Width	First lower molar	2.65 ± 0.03	2.43 ± 0.03 **	
Gingival PGE ₂ (pg/mg tissue)			148.70 ± 3.14	218.00 ± 23.60 *

*p<0.05, **p<0.01 and ***p<0.001 vs. control. ABL: alveolar bone loss. PGE₂: prostaglandin E₂. LPS: lipopolysaccharide.

*p<0.05, **p<0.01 and ***p<0.001 vs. control. ABL: alveolar bone loss. PGE₂: prostaglandin E₂. LPS: lipopolysaccharide.

whereas this increase was significantly prevented in HU 308-treated group (Table 4). The differences mentioned for the lingual side were not detectable on the buccal side of the mandibles, except for the difference between the control and LPS groups. In turn, when the whole tooth was evaluated, both upper and lower first molars evidenced the preventive effect of HU 308 treatment on alveolar bone loss, increased by lipopolysaccharide. In the same 3-week study, alveolar bone loss measured by the width method did not exhibit significant differences among control, early stage-experimental periodontitis and HU 308 early-stage experimental periodontitis groups in the lower first molar (Table 4).

• Histological analysis

In the analysis of the interradicular bone of the first lower molar, the early-stage experimental periodontitis and HU 308 early-stage experimental periodontitis groups exhibited an increase in the periodontal space compared to the control group, but did not differ significantly from each other

after the 3 week-treatment (Fig. 1A and B). On the other hand, the evaluation of the alveolar bone area, represented as B.Ar/T.Ar (%), revealed that early-stage experimental periodontitis rats had a lower value than control rats, while that reduction was prevented, at least partially, by the HU 308 treatment (Fig. 1C).

In the analysis of the interdental septum bone, the early-stage experimental periodontitis group revealed lower whole perimeter of the interdental septum, less bone formation areas, more bone resorption areas and higher quantity of osteoclasts than the control group. However, HU 308 treatment reversed all altered records, showing longer perimeter of whole septum, more formation areas, fewer resorption areas and fewer osteoclasts than the early stage-experimental periodontitis group (Fig. 2).

• Radioimmunoassay of prostaglandin E₂

Although early-stage experimental periodontitis caused a remarkable increase in prostaglandin E₂

Table 4. Macroscopic evaluation of alveolar bone loss measured by the distance method in the first upper (A) and lower (B) molar, for control, LPS and LPS+HU 308 groups.

Macroscopic ABL (mm)			Control	LPS	LPS + HU 308
Distance	First upper molar	Buccal	1.25 ± 0.16	1.51 ± 0.13	1.28 ± 0.16
		Palatal	1.27 ± 0.16	1.51 ± 0.22	1.22 ± 0.20
		Whole tooth	2.52 ± 0.09	3.02 ± 0.13 [*]	2.50 ± 0.11 [#]
	First lower molar	Buccal	1.08 ± 0.15	1.36 ± 0.20 [*]	1.25 ± 0.19
		Lingual	2.66 ± 0.21	3.70 ± 0.42 ^{***}	3.27 ± 0.32 ^{**}
		Whole tooth	3.74 ± 0.10	5.22 ± 0.14 ^{**}	4.52 ± 0.19 ^{**#}
Width	First lower molar		2.40 ± 0.04	2.34 ± 0.03	2.43 ± 0.04

ABL of buccal and palatal/lingual sides and whole tooth are shown for the first upper and lower molars. The width of the first lower molar is presented as a complementary evaluation.

^{*} $p < 0.05$, ^{**} $p < 0.01$ and ^{***} $p < 0.001$ vs. control; [#] $p < 0.05$ vs. LPS. ABL: alveolar bone loss. LPS: lipopolysaccharide. HU 308: synthetic agonist of the cannabinoid receptor 2.

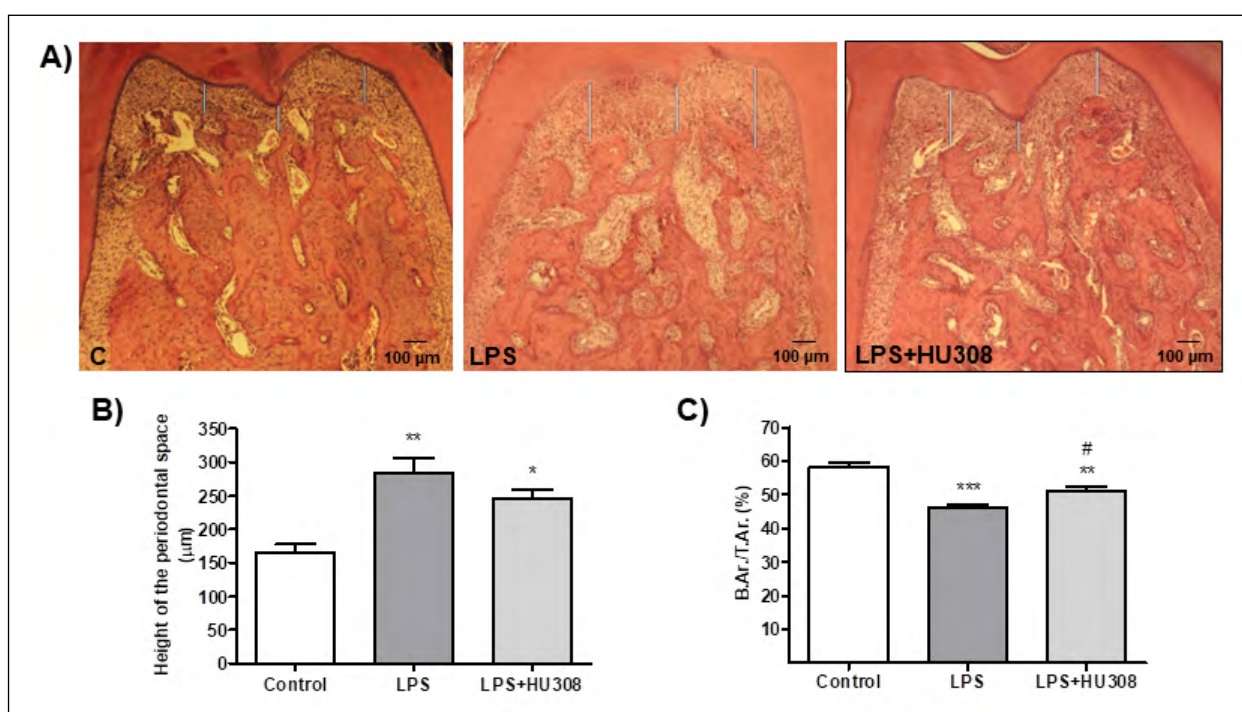


Fig. 1. A) Photomicrographs (H&E stain; original magnification $\times 40$) showing histologic features of the mandibular first molar interradicular area of rats subjected to different experimental conditions (control, LPS and LPS+HU 308). B) Periodontal space height evaluation. C) Interadicular bone measured as B.Ar/T.Ar (%). Results are presented as mean $\pm$ SEM. ^{*} $p < 0.05$, ^{**} $p < 0.01$ and ^{***} $p < 0.001$ vs. control; [#] $p < 0.05$ vs. LPS.

in comparison to controls, no significant difference was found between early-stage experimental periodontitis and HU 308-treated groups (Fig. 3). However, after the 3-week study period, a downward trend can be detected in the CB2r-agonist group with respect to early-stage experimental periodontitis.

DISCUSSION

Being one of the most prevalent disorders of the oral cavity, periodontal disease has been extensively

studied in laboratory animals. To this end, endotoxin LPS-induced periodontitis has been a method which aimed to achieve a more physiological process of the spontaneous disease than traumatic methods^{24,25}. The traumatic model consists of placing a ligature around molars and maintaining it, usually for two weeks, which produces a traumatic effect and facilitates dental plaque accumulation²⁶. In the LPS-induced model, toxin reaches periodontal tissues and no traumatic effect is generated other than the needle

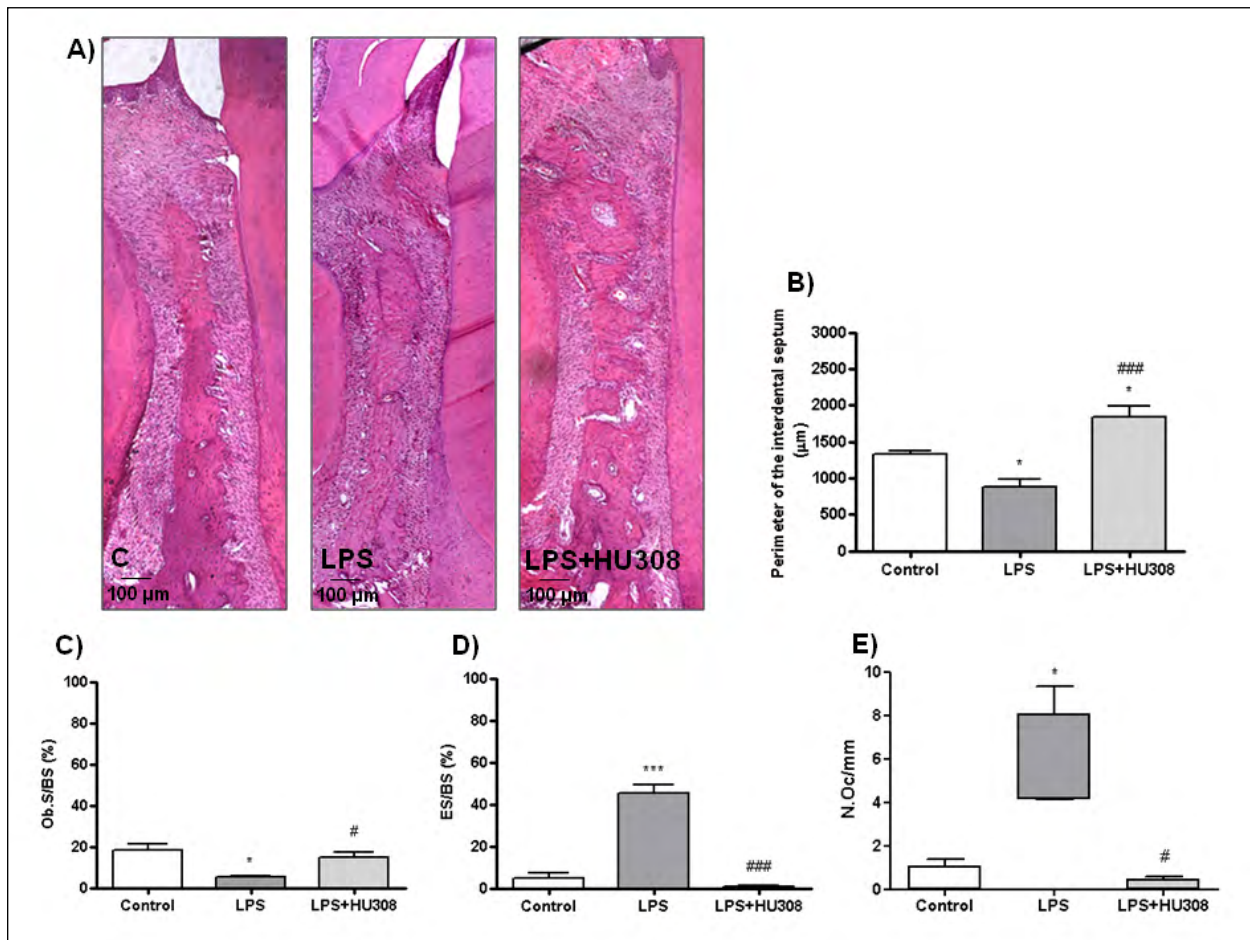


Fig. 2. A) Photomicrographs (H&E stain; original magnification $\times 100$) showing histologic features of the interdental septum bone located between the first and the second lower molars of rats subjected to different experimental conditions (control, LPS and LPS+HU 308). B) Whole perimeter of interdental septum bone expressed in μm . C) Bone formation surfaces expressed in % as osteoblast surface/total bone surface (Ob.S/BS). D) Bone resorption surfaces expressed in % as eroded surface/total bone surface (ES/BS). E) Number of osteoclasts expressed as N.Oc/mm. Results are presented as mean $\pm$ SEM. * $p < 0.05$ and *** $p < 0.001$ vs. control; # $p < 0.05$ and ### $p < 0.001$ vs. LPS.

injection. However, scientific literature has not reached a consensus with regard to the characteristics of this model or to the terms used for the induction. Indeed, different authors have run trials lasting 1 to 10 weeks^{24,25,27-30}. Moreover, in previous studies we have demonstrated that LPS injections in first molar gums for six weeks produce a set of chemical and anatomical changes in periodontal tissues^{14,16,31}. At this time, the increase in alveolar bone loss and inflammatory mediators such as $\text{TNF}\alpha$, PGE_2 and nitric oxide (NO) in gingival tissues indicate that EP has been established. Therefore, in the current study, we firstly focused on evaluating conditions of periodontal disease at several periods of time in order to clarify the emergence and severity of the damage at earlier terms than six weeks.

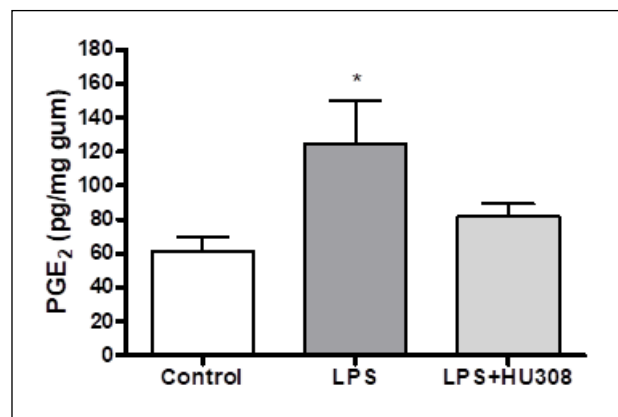


Fig. 3. Content of prostaglandin E_2 in gingival tissue: the effect of LPS and the application of HU 308. Results are presented as mean $\pm$ SEM. * $p < 0.05$ vs. control.

We found that no alveolar bone loss was induced by LPS injections after one week of the treatment, whereas after three weeks, the alveolar bone loss by the distance method was significantly higher in the LPS-injected group than in the control group. Furthermore, the difference between groups was even more pronounced after six weeks, when the alveolar bone loss was also evident from the diminished width. Regarding PGE_2 gingival content, results showed variability in control values among different times assessed in separate experiments, which could be associated with the high volatility and complicated biology of PGE_2 , in part due to the high number of PGE_2 synthesizer cells and their variable response to the presence of antigens³². Therefore, it is justified to analyse the results of each experiment separately. Despite these almost inevitable discrepancies, our results showed that gingival PGE_2 content was significantly higher in LPS-induced periodontitis than in control rats at each assessed time.

Overall, these findings suggest that in early stages of LPS induction, even when alveolar bone damage is not clearly marked, gingival tissue expresses some sign of inflammation, a stage which could be related to gingivitis in human populations³³. In addition, the first signs of alveolar bone loss at three weeks suggest that 3 weeks, or sometime between 1 and 3 weeks of LPS induction could be defined as the beginning of periodontitis in rats, though further studies are needed.

Since initial signs of alveolar bone loss were identified after three weeks, we subsequently assessed the effects of the HU 308 treatment at this time and considered it as an early stage of experimental periodontitis.

At 3-weeks, alveolar bone loss by the distance method increased on certain sides by LPS, was prevented in HU 308-treated rats. Some differences regarding control and LPS values between this study and the 3-week term assessed in the study time range might be caused by logical variability between experiments or due to random factors. In any case, distinct alveolar bone damage between control and LPS groups was also noticeable in this 3-week experiment, where even in group treated with HU 308, a mitigating effect emerged. In the histological analysis, the preventive effect of HU 308 on bone resorption was confirmed in the bone volume evaluation, even though a statistically non-

significant tendency in the same direction could also be observed in the periodontal space evaluation. These results are consistent with those we found in the 6-week treatment, where these initial tendencies appeared to be strengthened. In fact, after six weeks, LPS-induced damage was clearer on both sides of the first upper and lower molars, while the HU 308 treatment efficiently attenuated it¹⁶. Furthermore, another study using *in vitro* human periodontal ligament cells demonstrated that LPS increases the concentration of IL-1b, IL-6, TNF- α , and RANKL, while HU 308 attenuates these parameters, normalizing bone metabolism³⁴. On the other hand, histomorphometric measures on the interdental septum, such as perimeter of bone septum, bone formation areas, bone resorption areas and number of osteoclasts, lead to a scenario characterized by bone resorption in the LPS group and bone formation or resorption prevention in the HU 308-treated group. In this respect, in a recent study, Kamali et al. demonstrated the osteogenic effects of a scaffold based on cannabidiol, a natural cannabinoid, on bone defects in rats, employing histomorphometric measurements, among other techniques³⁵.

PGE_2 content evaluation in the gingival tissue showed a higher value in LPS-induced group with respect to controls, in agreement with the time range studies results. However, this increase was not significantly modified by the HU 308 treatment at three weeks, although a downward trend was evidenced. This result suggests that in a stage of emerging damage, HU 308 could start to show an incipient preventive effect, which is stronger and statistically significant at six weeks of induction²⁰. Similarly, in our previous six-week study, other inflammatory mediators such as TNF α and NO, which were increased in gums by LPS-induced periodontitis, also showed the reducing effect of the HU 308 treatment. Thus, attenuation of PGE_2 in gingival tissue reveals the therapeutic potential of the CB2r stimulation, providing a potential way to reduce inflammatory and immune responses as well as to prevent bone resorption, probably through the regulation of osteoblast and osteoclast activity³⁶.

In conclusion, a 3-week term of LPS-induced periodontitis is a valid model of the early stage of the disease, since emerging damage is expressed in bone tissue, although it is not as clear as in a 6-week term. Additionally, we demonstrated herein that 3-week harmful effects could be prevented by

local stimulation of CB2r with HU 308. Current outcomes shortly after the beginning of LPS-induced periodontitis also support the importance of initiating interventions in early stages of the

pathology, before greater damage is produced, thereby preventing irreversible alterations of periodontal health in humans.

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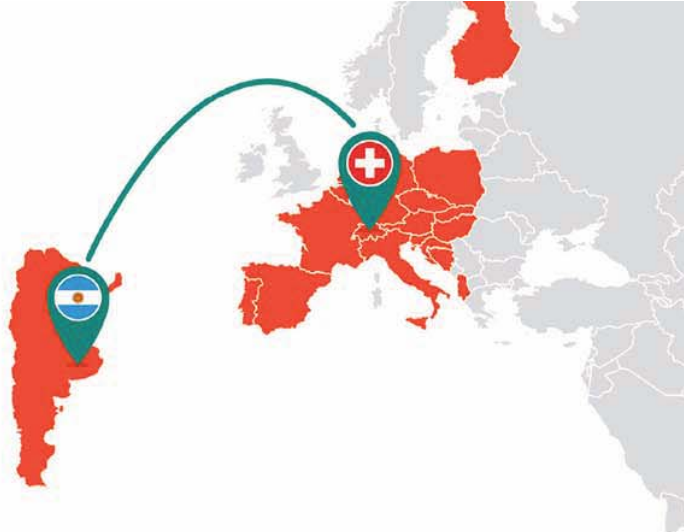


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