Original Article

Effect of resin infiltration on the nanomechanical properties of demineralized bovine enamel

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Abstract

Objective: The aim of the present study was to evaluate the efficacy of resin infiltration in preventing *in vitro* lesion progression. **Materials and Methods:** Buccal surfaces of bovine incisors were divided into mesial and distal regions and, at the center, nail varnish was applied (1.0 mm width) to protect the enamel surface against any further treatment. In order to create artificial enamel lesions in the unprotected areas, each specimen was soaked in a demineralizing solution. After that, specimens had two enamel lesions. One lesion in each sample was etched with 15% HCl for 120 s and infiltrated with a commercial infiltrating resin for 3 min, while the other lesion was not treated (control). Each specimen was cross-sectionally halved and randomly allocated to two groups: Group 1 was immediately processed and Group 2 was submitted to a new demineralization process. The samples were analyzed by means of cross-sectional hardness measurements using a nanoindenter equipment. Hardness data were statistically analyzed by non-parametric Kruskal–Wallis and MannWhitney tests ($\alpha = 0.05$). **Results:** The findings showed statistical difference between treatments at the same analyzed distance range from the outer surface of the enamel ($P < 0.05$). **Conclusion:** The untreated lesion showed lower hardness values for distances near the outer surface of the enamel. The resin infiltration was efficient in preventing further *in vitro* demineralization of bovine enamel lesions.

Key words: Dental caries, dental enamel, hardness

INTRODUCTION

Caries infiltration with low-viscosity resins has recently been considered an innovative attempt to treat lesions.[1-4] Indeed, the resin infiltration seems to be an efficient way to avoid further demineralization process of artificial and natural enamel lesions under cariogenic conditions.[3-5] Mechanical properties of enamel submitted to different treatments have been studied by means of cross-sectional hardness evaluation.[6-8] Resin infiltration significantly increased microhardness and reduced lesion progression as compared to untreated artificial lesions.[9] Nevertheless, lesions filled with commercial resins present low microhardness after a further acid etching, and some demineralization process can still occur after treatment.[10] However, the effect of lesion infiltration on hardness has not been clarified yet. The mechanical properties of the caries infiltration with low-viscosity resin should be affected by the degree of demineralization and penetration of the resin.

Nanoindentation tests have become a common technique for analyzing mechanical properties of small areas and thin layers.[6-8] In nanoindentation, one generally makes use of the instrumented indentation technique where load and penetration depth are two independent variables continuously measured during the tests, in contrast to the traditional methods of hardness measurements where the residual impression dimensions are used to obtain the hardness. The capability of performing measurements at very small scales has led the nanoindentation technique to be used for evaluating the mechanical properties of biological materials.[11] For example, the properties of bone have been studied regarding its mineral, collagen

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and water content.[12] As a matter of fact, the emerging nanoindentation methods have opened up new possibilities of studying the mechanical properties of mineralized hard tissues. However, many intrinsic (enamel, dentin, sheaths and prisms) and extrinsic factors (lesions, storage medium, etc.) related to teeth influence the nanoindentation measurements, making the interpretation data not trivial.[13] Even so, a closer investigation is desirable to achieve a better understanding of mineralized tissue properties.

In this work, cross-sectional hardness behavior of bovine enamel lesions filled with a commercial light-curing resin and untreated lesions were evaluated. Hardness was measured by nanoindentation tests at different spots as a function of the distance from the outer surface of the enamel toward the inner part of the tooth. It was hypothesized that the nanohardness of the untreated lesion is significantly reduced compared with the treatment group (infiltration) when submitted to a second demineralizing etching.

MATERIALS AND METHODS

Preparation of bovine teeth

Bovine teeth kept in 1% formaldehyde aqueous solution were employed in this study. The tooth crown cut at the cementoenamel junction was used as a specimen. After embedding in acrylic resin, the buccal surfaces were abraded with 400-grid to 1200-grid silicon carbide abrasive papers (Struers S/A, Struer, Denmark) in order to obtain flat enamel surfaces. Three specimens were chosen with a minimum surface area of 4.0 x 4.0 x 3.0 mm. The buccal surfaces of bovine incisors were divided into mesial and distal regions (according to Iijima *et al.*[8]). Two consecutive layers of acid-resistant nail varnish were applied at the center region of about 1.0 mm width [Figure 1]. The artificial lesions were created through immersion of the samples into an acidified gel (0.1 N lactic acid containing 500.0 mg/L hydroxyapatite and pH 4.6)[14] at 37°C for 72 h [Figure 1]. After the demineralization process, the samples were immediately thoroughly rinsed with distilled water for 1 min. In this way, the specimens

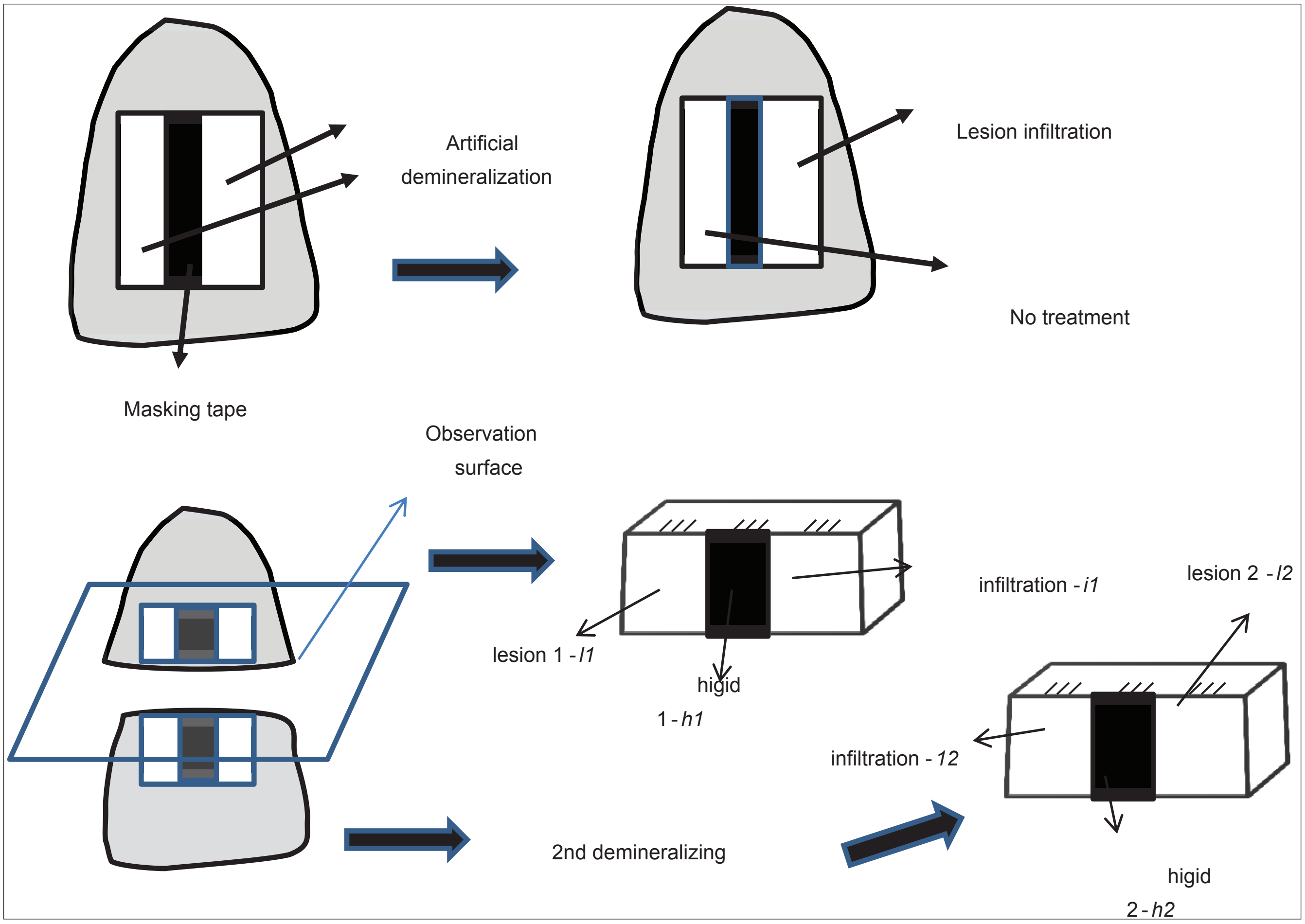

Figure 1: Schematics of the sample preparation steps

presented two enamel lesions. One of them was etched with 15% HCl for 120 s (Icon-Etch - DMG, Hamburg, Germany), rinsed with distilled water for 30 s and dried in air. Icon-dry was also applied for 30 s (air dried) and infiltrated with Icon® resin (DMG, Hamburg, Germany) for 3 min. Finally, samples were light-cured at 400 mW/cm² (Optilux model VCL 400 curing light Kerr Inc., Danbury, CT, USA) for 40 s. The other lesion was not infiltrated (untreated control).

After storing the specimens in distilled water for 24 h at 37°C, all teeth were cut with a slow-speed water-cooled diamond saw (ref. 7020, KG Sorensen, Barueri, SP, Brazil) and divided into incisal and cervical halves. One sectioned specimen (transverse planes) was then embedded in acrylic resin (Classico LTDA, São Paulo, SP, Brazil) and prepared for nanohardness testing (Group 1) and the other was submitted to a new caries process using the same demineralizing solution, as described above (Group 2), and then also prepared for nanohardness tests in the same way [Figure 1]. The cutting surface was covered with nail varnish before demineralization. Thus, the samples had three different areas in Group 1: Lesion (l1), higid enamel (h1) and infiltrated lesion (i1); and three areas in the Group 2: Lesion (l2), higid enamel (h2) and infiltrated lesion (i2). Finally, nanoindentation tests were carried out on the samples in the two groups, as detailed in the next section.

Nanoindentation tests

The cross-sectional specimens were mirror abraded with 400-grid to 1200-grid silicon carbide abrasive paper and then polished with 1.0 μm alumina suspension (Buehler Ltd, Lake Bluff, IL, USA).

Nanoindentation measurements were carried out by applying a normal maximum load of 4.0 mN, 0.27 mN/s load rate and 10s peak hold time using a nanoindenter equipment G200 (MTS/Agilent, Santa Clara, CA, USA). The method of Oliver and Pharr[15] was employed for hardness calculations.[15] A Poisson's coefficient of 0.2 was employed. A fused-silica sample with known properties was used for tip area calibration. A total of 90 measurements was performed on each of the three different areas: The lesion (l), nail varnish covered or higid (h) and Icon-infiltrated (i) areas. Nanoindentation tests were performed from the outermost surface of the enamel to the inner part of the enamel incisors starting at about 5.0 μm far from the enamel surface. Three lines with 30 indentations each were performed at intervals of 5 μm from each other perpendicularly to the enamel flat surface on each one of the three regions. The hardness results were then analyzed in terms of their distance from the outer surface (from 5.0 to 150.0 μm).

Statistical analysis

Hardness average values, their minimum and maximum values, as well as their standard deviations were obtained from nanoindentation tests for five different distance ranges (5-30, 35-60, 95-120 and 125-150 μm). Statistical analyses were performed by means of Statgraphics Centurion 5.1 software (StatPoint Technologies Inc., Warreton, VA, USA). After checking the error distribution and variance homogeneity by the Shapiro–Wilk and Levene tests, respectively, the hardness data were analyzed by the non-parametric Kruskal–Wallis and Mann–Whitney tests. A significance level of $\alpha = 0.05$ was employed.

RESULTS

Figure 2 shows the typical load-displacement curves obtained from nanoindentation tests carried out on higid enamel (*h1*), lesion (*l1*) and lesion infiltrated with Icon® resin (*i1*; Group 1 samples). In Figures 3 and 4, the obtained average hardness (h) values are plotted as a function of the distance from the outer surface of the enamel for the Group 1 and 2 samples. Both plots present large standard deviation bars at distances near the outer surface of the enamel (<60 μm).

Table 1 displays the statistical analysis of the results. The non-parametric Kruskal–Wallis test showed that there are significant differences in the nanohardness average values between the Groups 1 and 2 samples ($P < 0.05$). Hardness of the untreated lesions decreases significantly after second

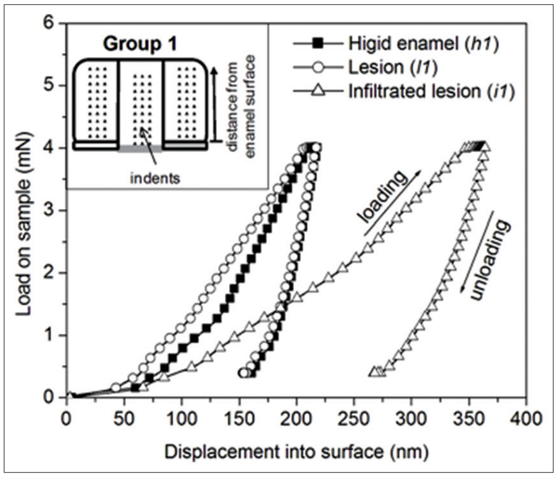

Figure 2: Typical load versus displacement curves obtained from nanoindentation tests for higid enamel (*h1*), lesion (*l1*) and lesion infiltrated with Icon® light-curing resin (*i1*) samples. Measurements were carried out 5 μm away from the enamel surface, under a load of 4.0 mN. The inset depicts the indent's location on each region of the sample. The direction of the indentation is normal to the plane of the figure

Table 1: Statistical analysis for samples from Groups 1 and 2 (hardness mean values, minimum and maximum values, standard deviations)

Group	Sample	Distance range from the outer enamel surface (μm)					
		5-30	35-60	65-90	95-120	125-150	Total
1	Higid enamel (*h1*)	4.84 (0.30)$^{A*\#}$ 4.12-5.24	4.80 (0.53)$^{*\#}$ 4.15-5.87	4.65 (0.76) 4.29-7.53	4.45 (0.29) 3.95-4.83	4.38 (0.31) 3.75-5.07	4.57 (0.43) 3.75-7.53
	Lesion (*l1*)	4.09 (0.59)$^{A*\#}$ 3.14-6.52	4.52 (0.69)$^{*\#}$ 3.80-5.91	4.73 (0.67)* 3.64-7.06	4.39 (0.39)^{A*} 3.56-5.37	4.35 (0.29) 3.50-5.11	4.44 (0.56) 3.14-7.06
	Infiltrated lesion (*i1*)	4.21 (1.02) 1.23-6.84	4.44 (0.85) 3.24-5.68	4.50 (0.45) 3.55-5.13	4.42 (0.48) 3.69-5.61	4.14 (0.45) 3.55-5.13	4.35 (0.64) 1.23-6.84
2	Higid enamel (*h2*)	4.48 (0.51)$^{B\#}$ 3.88-5.69	4.04 (0.43) 3.73-5.97	3.97 (0.47) 2.63-4.62	3.96 (0.34) 3.27-4.63	3.73 (0.52) 3.77-4.96	3.98 (0.75) 2.63-5.97
	Lesion (*l2*)	3.62 (0.37)$^{B\#}$ 2.73-4.27	3.99 (0.35)$^{\#}$ 3.07-5.01	4.02 (0.31)$^{\#}$ 2.97-4.53	4.01 (0.43)$^{B\#}$ 3.04-4.70	3.79 (0.50) 0.86-4.85	3.90 (0.41) 0.86-5.01
	Infiltrated lesion (*i2*)	3.90 (1.27) 2.02-5.72	4.36 (0.65)$^{\#}$ 3.14-5.48	4.38 (0.40)$^{\#}$ 3.78-5.41	4.38 (0.34)$^{\#}$ 4.01-5.47	4.34 (0.36) 3.95-5.26	4.32 (0.66) 2.02-5.82

Different letters mean statistical differences between groups for the same treatment and distance. ($^{\#}$) Statistical differences between treatments in the same group. (*) Statistical differences between treatments in the same groups in the distances measured ($P<0.05$) A,B=$P<0.05$

demineralization etching when compared with the infiltrated lesions ($P < 0.05$).

DISCUSSION

Mechanical properties of the enamel can be affected by the demineralization rate, biological differences among teeth (and patients), resin penetration depth in the enamel, storage medium and hydration state of the samples.[8,9,13] Microhardness tests have been used to investigate the hardness changes of the enamel surfaces when submitted to treatments that affect their mechanical properties.[9] In addition, cross-sectional nanohardness tests were used to evaluate how deep such changes occur in the enamel layer.[6,8] Nanohardness indentation measurements can provide indirect evidence of mineral loss or gain.[6,8] Variations in mechanical properties for the different domains of enamel, therefore, might be of importance for understanding mechanisms and therapeutic strategies as well as novel biomimetic materials designs structure.[8,16-19]

Enamel caries lesions are characterized by a loss of mineral in the lesion body, whereas the surface remains comparably highly mineralized. In this study, the average hardness values were lower for the lesion areas, as shown in Table 1 *(l1* - Group 1 samples). Such results indicated that mechanical degradation occurred due to the demineralizing process; however, they do not seem to show a more mineralized surface area. This may be attributable to the fact that mechanical properties do not change linearly with the degree of mineralization (6 days exposure to acid); hence, the small reduction in mineral content that occurs during early exposure to acid may have a negligible effect on mechanical properties.[6] After the second demineralization process (6-days exposure to acid), there is a surface zone much more mechanically degraded (*l2* – Group 2). On the other hand, the infiltrated artificial lesion (i1 sample) tends to present slightly lower hardness compared with the higid enamel (*h1* sample) and not different from the lesion group (*l1* - Group 1 samples). In Group 2, the untreated lesion (*l2* sample) decreased hardness after demineralization as compared to the infiltrated lesion (*i2* sample). In contrast, Paris *et al.*[9] (2013) have found that resin infiltration (Icon®) significantly increased the transversal microhardness and reduced the lesion progression when compared with untreated artificial lesions after demineralization.[9] Such apparently contradictory findings could be related to the methodology here applied. Paris *et al.*[9] (2013) measured the microhardness starting at 50 μm from the enamel outer surface, while our measurements started at about 5 μm from the enamel surface; therefore, our results show the mechanical (hardness) changes at the shallowest depths more accurately. Thus, nanohardness is a sensitive technique for shallow lesions, provided that the lesions are less than 50 μm at any time during the study.

Furthermore, the experimental protocol for caries infiltration requires HCl application for 120 s for ensuring the entire resin infiltration in the lesion.[20] Regarding the main features of the produced artificial caries, lesions were partially removed and infiltrated (at shallow depths). The infiltrated lesions presented a slight increase in hardness after infiltration in the outmost surface, but such increase did not take place in the inner parts (Group 1, *1i*). After the second demineralization process, the infiltrated lesions did not have notable mechanical changes as the untreated lesions, presenting hardness values close to the control group (Group 1- *li*). This finding confirms many studies that have shown that untreated lesions accelerated when exposed to a demineralizing condition and

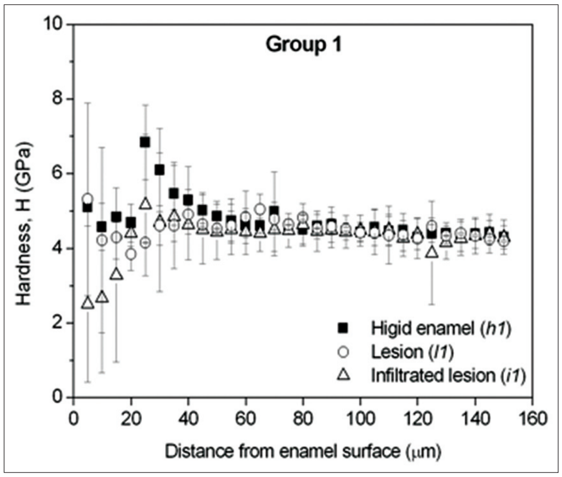

Figure 3: Average hardness (H) as a function of distance from the outer surface of the enamel obtained by nanoindentation tests for the Group 1 (control) samples: Higid enamel (*h1*), lesion (*l1*) and lesion infiltrated with Icon® light-curing resin (*i1*) samples. Load of 4.0 mN; *n* = 9

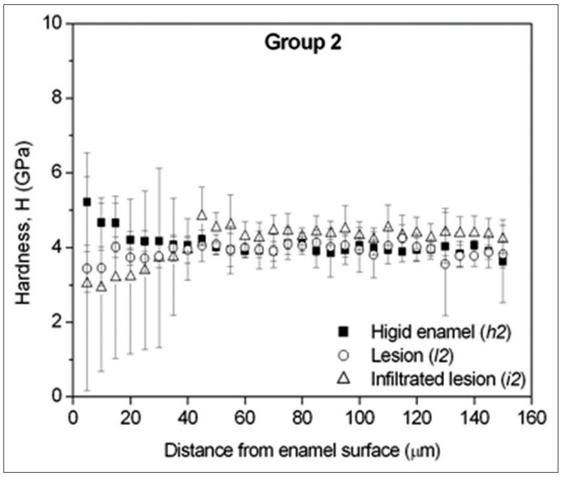

Figure 4: Average hardness (H) as a function of distance from the outer surface of the enamel obtained by nanoindentation tests for Group 2 samples: Higid enamel (*h2*), lesion (*l2*) and lesion infiltrated with Icon® light-curing resin (*i2*) samples. Load of 4.0 mN; *n* = 9

infiltrated lesion is efficacious in preventing the further progression of artificial lesion.[3-5,21-23]

A small mechanical degradation took place in the enamel higid group (Group 2- *h2*). Probably, the nail varnish did not seal completely the enamel surfaces, thereby provoking demineralization at certain points on the surfaces.

A previous study has reported an increase of surface Vickers microhardness when infiltrating resins were applied.[10] This reflects the ability of the low-viscosity resin to fill the spaces between the remaining crystals of the porous lesion and the demineralized tissue, improving the mechanical strength of the infiltrated enamel surface. The low-viscosity Icon® resin has a high TEDGMA monomer concentration, which is hydrophobic, and absence of water is a requirement for lesion infiltration. This is obtained by applying ethanol for 30 s. Usually, such resin materials are not capable of infiltrating the lesions when water is present.[10] Also, Torres *et al*.[10] (2012) have observed that lesions infiltrated with Icon® resin decreased the microhardness of the enamel surface, not presenting any difference to the untreated enamel and fluoride-treated samples after being submitted to a new acid challenge.[10] Therefore, unfilled areas at the bottom of the lesions become susceptible to further lesion.[20]

In the present work, bovine enamel was used as a substitute for human enamel. Other studies have also used bovine enamel for similar purposes.[3,7,10,20] In general, bovine enamel surfaces have a wide range of hardness values, as seen in Figures 3 and 4. Surface porosity strongly affects the nanoindentation tests once the indenter tip can slide into the pores; additionally, the bovine enamel is usually more porous than the human one.[7,20] In Table 1 (*l1* - Group 1 samples), the data show that hardness decreased when the lesion process occurred. When the enamel surface was filled with infiltrating resin, a larger standard deviation variation is observed, mainly at the outer enamel surface [Figure 4]. Thus, the pre-treatment in HCl solution could have influenced our findings, increasing the enamel dissolution and, consequently, changing its hardness measured by nanoindentation tests. Belli *et al*.[20] (2011) have also observed that bovine enamel has macroscopic-sized voids that can accelerate the caries process, thereby being harder to be filled by the resin infiltration. Therefore, the enamel can present demineralized zones unfilled by the infiltrating resin, which have poor mechanical properties; meanwhile, it can present high mineral content zones or resin-filled areas, which are mechanically more resistant. Such effects could also support the hardness values variation found herein.

Recently, H_3PO_4 solutions are widely used in laboratory tests due to the limitations imposed by artificial caries processes, such as the shallower depths compared with the natural lesions.[9] Thus, immersion in 37% H_3PO_4 solution for 5 s removes the outer surface layer and, therefore, seems to be suitable for the treatment of artificial enamel lesions prior to the resin infiltration. Nevertheless, previous studies reported that 15% HCl is also effective for the infiltration technique using bovine enamel.[3,10,20]

Therefore, the infiltrated resin protects the lesion against further exposure to the demineralizing solution but reduces the hardness values to a certain extent, as seen in Table 1 (*i2* – Group 2 samples). Therefore, despite reducing the demineralization, some demineralizing process could have already occurred in the Icon® resin-infiltrated area.[2,4,23] This effect could be minimized by further resin application. In our case, only one resin application was accomplished; on the other hand, an additional resin application appears to increase the hardness and enhance the demineralization resistance.[9] Torres *et al*.[10] (2012) have also reported a surface hardness reduction when infiltrated lesions were submitted to a new cariogenic solution. Accordingly, this effect can occur due to some enamel dissolution that is not entirely impregnated by the adhesive or, thus, to crack nucleation on the surface during the photo-curing procedure.[10]

Differing from the microhardness tests, nanoindentation ones refer to depth-sensing indentation tests carried out at the submicron range, in which very small indentations are made while the load and displacement are recorded with higher accuracy.[8] The load-displacement curves from nanoindentation tests can present discontinuities on depth penetration (called pop-ins), which are commonly associated with fracture mechanisms.[15] This is the case of crack propagation during loading in brittle ceramics. Here, pop-ins were not observed during the indentation tests, suggesting that the samples were not fractured during loading [Figure 2]; i.e. the analyzed surfaces presented merely elastoplastic behavior and tolerated the contact pressures imposed during tests.

Our study investigated one infiltrating resin material under *in vitro* conditions, which obviously do not completely mimic the oral cavity environment. Based on the nanoindentation results, the infiltrating resin was able to protect the region against further demineralization process, despite tending to reduce the hardness values slightly.

CONCLUSIONS

In this work, demineralized bovine enamel was infiltrated with a commercial dental resin. The nanoindentation technique was employed to investigate the hardness variation of cross-sectional surfaces produced by *in vitro* demineralization. The untreated lesions had the lowest hardness values for distances near the outer surfaces of the enamel. Resin infiltration appeared to be efficient at preventing further demineralization of enamel lesions under the laboratory conditions used.

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