Evaluation of the effects of the bonding agent on acid-etched human enamel demineralization: *in situ* study

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SUMMARY This *in situ* study evaluated the influence of the bonding agent on demineralization of acid-etched human enamel. Enamel blocks obtained from 20 human molars (four blocks per tooth) were divided into five groups. For the control groups, healthy control (HC, no treatment) and acid-etched control (EC, etching with 35 per cent phosphoric acid for 20 seconds), the specimens were obtained by sectioning in half, one block from each tooth. For the experimental groups, experimental etched group (EE), experimental Concise™ group (CE), and experimental Transbond™ group (TE), all specimens were etched with 35 per cent phosphoric acid for 20 seconds (EE) and treated with Concise™ enamel bond (CE) and Transbond™ XT primer (TE). Specimens from the experimental groups were fixed at intra-oral appliances wore by 10 volunteers and exposed to oral environment. After 28 days, the specimens were sectioned in the mesial–distal direction and the enamel cross-sectional Knoop hardness number (KHN, 50 g, 15 seconds) was measured at the cervical and occlusal regions at 25, 50, and 75 μm from the enamel surfaces. Data were analysed by analysis of variance (ANOVA) and Tukey honestly significantly different test for multiple comparisons ($\alpha = 0.05$). Enamel KHN ($P < 0.05$) was recovered only in the Concise-treated group (CE; $P < 0.05$). When considering depth measurements, KHN results were 25 > 50 > 75 μm ($P < 0.05$). None of the groups exposed to the intra-oral environment for 28 days have recovered completely the enamel microhardness, but the order of improvement was better in the CE group than the TE group, the latter being only slightly better than the EE group.

Introduction

Composite resins are mainly used for bonding brackets in Orthodontics. The literature shows the advantages obtained when using these materials, justifying their wide application. Nevertheless, despite these considerations, it has been shown that there is more enamel demineralization around brackets bonded with these materials than with glass ionomer cements (Vorhies *et al.*, 1998; Gorton and Featherstone, 2003; Sudjalim *et al.*, 2007). Moreover, it is difficult to achieve a good oral hygiene around orthodontic appliances, increasing plaque retention, which put patients at a higher risk for developing enamel lesions adjacent to these appliances (Mizrahi, 1982; Vorhies *et al.*, 1998; Chang *et al.*, 1999). Furthermore, the presence of fluoride either from dentifrice usage or released from a dental material may significantly interfere with the progression of caries lesions around orthodontic brackets *in vivo* and *in vitro* (O'Reilly and Featherstone, 1987; Øgaard *et al.*, 1988a,b; Hu and Featherstone, 2005; Behnan *et al.*, 2010). The results of another study showed that using fluoride-releasing glass ionomer cement for bonding orthodontic brackets successfully inhibited caries *in vivo* (Gorton and Featherstone, 2003).

Several studies have assessed the demineralization around brackets and the influence of the orthodontics adhesives (Lehman *et al.*, 1981; Hu and Featherstone, 2005; Farrow *et al.*, 2007; Chin *et al.*, 2009; Ghiz *et al.*, 2009). In the direct bonding technique of orthodontic accessories to tooth surfaces, when enamel etching is performed, 'demineralized' areas that are not covered by the bonding agent and/or accessories are likely to remain exposed to the oral environment. In addition, increased roughness turns these surfaces susceptible to plaque accumulation (Collys *et al.*, 1993). As a preventive measure, this area could be sealed with a bonding agent; however, studies have shown that this procedure is not effective as a result of the effects of oxygen inhibition during polymerization of the adhesive. Both light- and chemically cured materials are susceptible (Ceen and Gwinnett, 1980). Other authors have reported that despite the absence of polymerization in the most external layer, tags are formed more deeply, protecting the enamel from demineralization and significantly decreasing the diffusion through dental enamel (Kuhar *et al.*, 1999; Patrick *et al.*, 2004; Hu and Featherstone, 2005; Paris *et al.*, 2006). Furthermore, Farrow *et al.*, (2007) found no significant reduction in the incidence of decalcification after prophylactic sealing around orthodontic brackets with an unfilled sealant or a filled flowable composite restorative material.

Based on the above considerations, this *in situ* study evaluated the influence of the bonding agent on demineralization of etched human enamel, using the Knoop microhardness test as a method of evaluation. Hardness as measured by penetration is a measure of the mechanical resilience of enamel and used in demineralization and remineralization studies (Delbem *et al.*, 2009).

Methods and materials

The study was approved by the Research Ethics Committee of the Fluminense Federal University School of Dentistry (Process CMM-HUAP n° 06/02). Ten healthy adult volunteers, range 23–41 years (mean age of 25.3 years, SD 8.1), were recruited among dentists and postgraduate students at the Fluminense Federal University, School of Dentistry, Niterói, Rio de Janeiro, Brazil, according to Featherstone and Zero (Featherstone and Zero, 1992) criteria and informed consent was obtained from all participants after they received oral and written instructions about the study. Volunteers lived in a city with a fluoridated water supply (0.7 mg F/l).

Preparation of enamel specimens

Twenty freshly extracted impacted human permanent third molars were used in this study. They were free of caries and fluorotic or hypomineralized lesions and other visible enamel defects and were stored and sterilized in 2 per cent formaldehyde solution, pH 7.0, at room temperature, for 30 days. Then, four enamel blocks were obtained from each tooth (Figure 1). A block, randomly chosen, was divided into two: one for the healthy control group (HC, no treatment) and the other for the etched control (EC) group. The other blocks were also randomly distributed to the experimental groups (Figure 1). They were submitted to prophylaxis with pumice and water. For each group, one enamel block was randomly chosen from each tooth, and thus, the groups contained 20 blocks each. In order to apply the test material to the occlusal and cervical enamel portion of each block, simulating the area that would be exposed after bonding a bracket, a pre-defined area was covered with a piece of adhesive tape measuring 1 mm in diameter, placed in the geometrical centre of the enamel block.

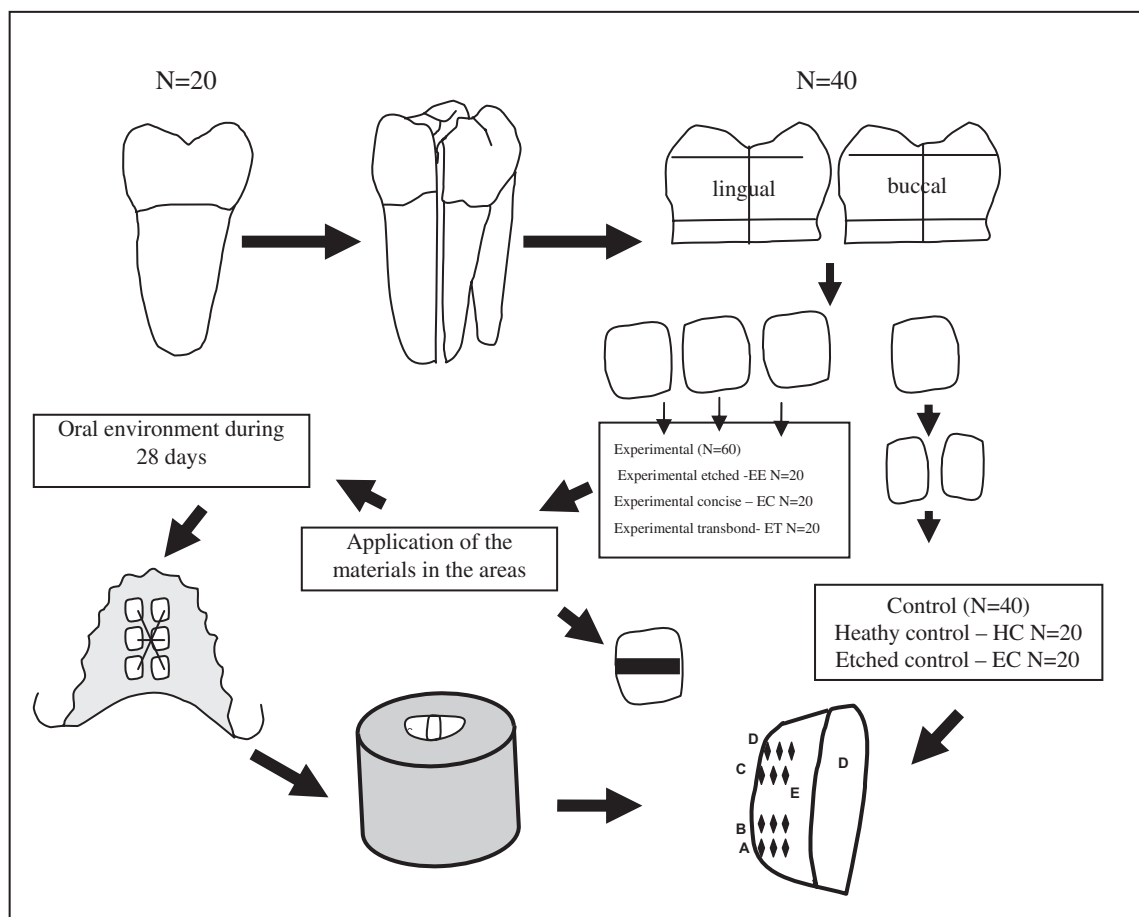The test material was applied around this area, and afterwards, a diamond bur (No. 2200; KG Sorensen™,

Figure 1 Illustration of the experimental design. E, enamel; D, dentin (A and B, cervical area and C and D, occlusal area). The first penetration at 25 µm, the second at 50 µm, and the last at 75 µm from the outer margin of the enamel.

Barueri, São Paulo, Brazil) was used to make a groove in the enamel, near the outer edges of the tape in order to identify the test area and to create a guide for the microhardness analysis. All samples were sterilized with ethylene oxide before receiving the material selected for each group.

The specimens were randomly divided into five groups: group 'HC': in this group, prophylaxis with a rubber cup, pumice, and water was performed for 15 seconds, followed by 10 seconds of water and air spray with triple syringe, and specimens were not subjected to the oral environment exposure. Group 'EC': prophylaxis with pumice and water for 15 seconds, followed by 10 seconds of water and air spray with triple syringe. Then, 35 per cent phosphoric acid gel (Transbond™ XT etching gel; 3M UNITEK, Moronvia, California, USA) was applied for 20 seconds, followed by 20 and 30 seconds of water and air spray, respectively. This group was immediately evaluated and it was not subjected to oral environment exposure. Group 'EE' (experimental etched): the same procedures were performed as those in Group EC, but it was exposed to the oral environment. Group 'CE' (experimental Concise™): similar to Group EC, but after the prophylaxis procedure and acid conditioning as described previously, a single layer of self-curing enamel bond resin A and B (Orthodontic Concise™; 3M UNITEK) was applied to the test area for 30 seconds and exposed to the oral environment. Group 'TE' (experimental Transbond™): similar to Group EC, but after the prophylaxis procedure and acid conditioning as described previously, a single layer of light-cured primer (Transbond XT™ Primer; 3M UNITEK) was applied to the test area for 30 seconds. Then, it was light-cured for 20 seconds at 400 mW/cm^2 (Optilux 400; Demetron Research Corporation, Danbury, Connecticut, USA). This group was exposed to the oral environment.

In situ phase

This was an *in situ* crossover study performed in a single phase of 28 days. Each volunteer wore samples from two teeth, i.e. the same volunteer wore six samples, two from each experimental group. The volunteers received instructions to wear the appliances continuously, even at night, but to remove them during meals (3 times/day). They were instructed not to brush the place in the intra-oral device where the samples were fixed. Throughout the experimental period, all volunteers used fluoridated toothpaste provided by the author (1100 ppm F as monofluorophosphate; Sorriso, Colgate-Palmolive, São Bernardo do Campo, São Paulo, Brazil).

Hardness measurement

At the end of the experimental phase, the specimens were removed from the appliance and immersed in 5 per cent sodium hypochlorite for 1 hour to remove debris and bacterial biofilms. The samples' evaluation from each group was made by analysing the enamel microhardness using a hardness tester (Micromet, 2003 series microhardness tester; Buehler™, Lake Bluff, Illinois, USA) with a Knoop diamond under a 50 g load for 15 seconds (Delbem *et al.*, 2009). The measurements were done by only one examiner, the first author. All blocks were longitudinally sectioned through the centre of the exposed enamel. In order to measure cross-sectional hardness, one-half of each block was embedded in acrylic resin, and the cut surfaces were exposed and polished (Figure 1).

Four rows with three indentations each were made. The rows were then identified: the first and more cervical—row A, the second—row B, the third—row C, and the more occlusal—row D. The indentations were made at 25, 50, and 75 µm from the outer enamel surface. The mean values of all four measurements points at each distance from the surface were then averaged (Figure 1).

Statistical analysis

Statistical analysis was performed using Statgraphics 5.1 Software (Manugistics, Rockville, Maryland, USA). The normal distribution of the errors and the assumptions of equality of variances were checked by Shapiro–Wilk and Levene tests, respectively. Since the assumptions were satisfied, the Knoop hardness number (KHN) data were analysed by three-way analysis of variance (ANOVA) and Tukey's honestly significantly different (HSD) test for multiple comparisons. The significance level was set at 5 per cent.

Results

The overall KHN results are shown in Table 1. Three-way ANOVA identified statistical significance for the three independent factors (group, region, and depth, $P < 0.01$). On the other hand, no significance was found for the double and triple interactions among them ($P > 0.05$). The results of Tukey's HSD test related to the groups are presented in Table 2. Table 3 summarizes the mean comparisons between the regions.

Discussion

The present *in situ* crossover study model has been used in many researches to assess the effectiveness of dental products used in the oral cavity and to evaluate the components used in the de-remineralization process (Featherstone and Zero, 1992; Stookey *et al.*, 1992). Microhardness analysis has been widely applied to evaluate the effect of de–remineralization on human enamel and provides useful information on mechanical properties (Lehman *et al.*, 1981; Collys *et al.*, 1993; Hu and Featherstone, 2005; Delbem *et al.*, 2009).

The mean microhardness of sound enamel was lower at the cervical region than at the occlusal region. It was

Table 1 Mean values of Knoop hardness number (KHN; standard deviations) for all groups. HC, healthy control; EC, etched control; EE, experimental etched group; CE, experimental Concise™ group; TE, experimental Transbond™ group.

Row	HC			EC			EE			CE			TE		
	Measurement depth from the specimen surface (µm)														
	25	50	75	25	50	75	25	50	75	25	50	75	25	50	75
Cervical region															
A	236.6 (51.3)	262.7 (49.4)	294.6 (42.7)	195.4 (51.4)	234.9 (41.9)	268.8 (55.3)	213.3 (58.3)	235.8 (52.3)	252.8 (55.9)	224.3 (49.5)	265.8 (45.2)	281.1 (41.5)	217.5 (57.3)	218.6 (72.3)	243.4 (39.9)
B	251.1 (43.8)	281.7 (38.4)	280.9 (31.5)	195.0 (52.3)	236.6 (52.9)	264.6 (45.3)	224.2 (66.4)	252.4 (43.0)	263.0 (50.6)	245.2 (41.1)	252.6 (47.0)	277.7 (48.3)	220.1 (57.4)	240.0 (60.6)	254.9 (36.5)
Occlusal region															
C	251.3 (44.5)	282.7 (49.8)	290.3 (63.3)	247.4 (35.8)	261.1 (37.0)	271.1 (34.3)	216.2 (57.9)	248.7 (59.0)	265.7 (64.1)	235.9 (70.8)	260.7 (56.5)	270.4 (44.9)	239.0 (41.0)	251.5 (39.8)	279.3 (41.2)
D	259.2 (55.5)	288.9 (42.5)	285.7 (42.7)	250.1 (42.2)	259.4 (30.4)	290.6 (56.2)	227.4 (58.0)	262.2 (57.7)	280.1 (50.7)	240.0 (76.5)	277.3 (50.7)	293.3 (35.1)	242.4 (49.9)	262.8 (47.1)	288.0 (38.4)

Table 2 Mean values of Knoop hardness number (KHN; standard deviations) of control and experimental groups. HC, healthy control; EC, etched control; EE, experimental etched group; CE, experimental Concise™ group; TE, experimental Transbond™ group.

HC	EC	EE	CE	TE
272.1 (49.4)[a]	247.8 (54.2)[b,c]	245.1 (59.6)[c]	260.3 (54.5)[a,b]	246.4 (52.5)[c]

Values with the same superscript letters are not statistically different ($\alpha < 0.05$).

Table 3 Mean values of Knoop hardness number (KHN; standard deviations) in the cervical and occlusal regions.

Cervical		Occlusal	
Row A	Row B	Row C	Row D
243.0 (56.6)[a]	249.3 (53.7)[a,b]	258.1 (53.2)[b,c]	267.1 (53.8)[c]

Values with the same superscript letters are not statistically different ($\alpha < 0.05$).

observed that the values increased gradually towards the occlusal and that difference was statistically significant when the mean hardness of the cervical region was compared to the occlusal and vice versa (Tables 1 and 3). In the group with acid etching, about 10 per cent of average hardness was lost. Lehman *et al.* (1981) found a reduction in values of microhardness after etching the enamel and a recovery in the presence of fluoride, whereas Collys *et al.* (1993) observed a recovery of microhardness when the etched enamel was exposed to the oral environment in the presence of fluoride. However, in the present study, it did not happen, i.e. there was no recovery of microhardness when the etched enamel was exposed to the oral environment, even though the volunteers brushed their teeth with fluoridated toothpaste. However, they did not brush the enamel samples. This result was supported by O'Reilly and Featherstone (1987), who observed enamel demineralization adjacent to brackets from patients who brushed their teeth with fluoridated toothpaste three times a day but showed bacterial biofilm accumulation.

The enamel microhardness value at the cervical region was lower than the one at the occlusal surface and this finding is of great clinical significance since decalcifications occur more frequently in these areas, and the present results showed that enamel microhardness would be even lower after enamel etching. Arakawa *et al.* (1979) reported that the enamel at the cervical areas of premolars and molars is prismless, and this interferes with the bonding of orthodontic accessories. It was observed in

the present study that there was lower hardness at the cervical area, regardless of the group, and as the accumulation of bacterial biofilm is greater in these areas, the demineralization process could be accelerated under unfavourable clinical conditions. In an *in vivo* study, O'Reilly and Featherstone (1987) found a 12 per cent loss of mineral content at the occlusal and 14 per cent at the cervical regions and at 25 μm from the enamel margin, after bonding, and these results are in agreement with the data obtained.

An increase in hardness was observed in the CE when compared to the EE. On the other hand, in the TE, the hardness value did not differ statistically from that of the EE group (Table 3). Concise™ is a self-curing material and Transbond™ is a light-curing material. Furthermore, this increase in hardness can be also attributed to the presence of tags in this outermost layer (Collys *et al.*, 1993; Glasspoole *et al.*, 1999; Kuhar *et al.*, 1999). When comparing the mean values of the control group, it was observed that the mean values obtained at 25 μm from the margin were lower than those obtained at 50 and at 75 μm, respectively, in all groups. However, only the CE showed recovery of hardness at all distances, which reinforces the idea that this increase is due to tag formation. The first explanation for these results can be based on differences between the material compositions. Concise™ is a Bisfenol Glicidil diMetAcrilatoor (Bis-GMA)-based self-curing material and Transbond™ is a tetraethyleneglycol dimethacrylate (TEGDMA)-BIS-GMA-based light-curing material. First of all, it is well known that Bis-GMA is more resistant to degradation hydrolysis than TEGDMA. Additionally, the presence of two aromatic rings in its molecule makes this monomer stiffer than TEGDMA (Gonçalves *et al.*, 2008). Thus, it is possible that the hybrid enamel below the Concise™ layer had been more effectively protected against *in situ* degradation, thereby presenting a higher hardness than the enamel hybridized by TEGDMA from Transbond™.

Several studies have shown that under favourable conditions, the etched enamel could be remineralized, especially in the presence of fluoride (Geiger *et al.*, 1988; Øgaard *et al.*, 1988). Hu and Featherstone (2005) observed that teeth treated with fluoride varnish exhibited 30 per cent less demineralization than the control teeth, the enamel-etched teeth, and the teeth treated with a light-cured unfilled sealant. In this study, despite the lack of restriction to the use of fluoridated toothpaste, it was encouraged to create a mechanism to accumulate biofilm, which would simulate the situation that occurs when an orthodontic accessory is bonded to the tooth. Therefore, under the conditions of the experiment, 28 days would be sufficient time to observe changes in the enamel by means of microhardness in the longitudinal section. Even under these conditions, the microhardness of the etched enamel exposed to the oral environment was not completely recovered, and its value was below the mean of the sound enamel and EC. Table 1

shows that lower values of microhardness were found in 5 of 6 measurements made at the occlusal region between groups EC and EE; however, there is no statistical significance between them (EC = EE, Table 2). According to Garberoglio and Cozzani (1979), some months are needed for the enamel to return to its normal condition after acid etching.

Several studies have assessed the demineralization of the etched enamel with phosphoric acid, with or without previous carious lesions and covered with adhesive or resin sealants (Van Dorp and Ten Cate, 1987; Kuhar *et al.*, 1999; Gorton and Featherstone, 2003; Hu and Featherstone, 2005; Farrow *et al.*, 2007; Sudjalim *et al.*, 2007; Ghiz *et al.*, 2009; Behnan *et al.*, 2010; Lodaya *et al.*, 2011). Paris *et al.* (2006) concluded that not only a deep infiltration but also a homogeneous resin layer prevents demineralization. Patrick *et al.* (2004) observed an average tag depth of 68 ± 22 μm, with the depth being greater in the demineralized enamel. Other studies have shown evidence that etched enamel impregnated with a bonding agent has lower permeability, but this protection decreased linearly with time (Van Dorp and Ten Cate, 1987; Kuhar *et al.*, 1999). Hu and Featherstone (2005) observed that filled sealant results in a significant reduction of enamel demineralization more than unfilled sealant. Farrow *et al.* (2007) found no significant reduction in the incidence of decalcification after prophylactic sealing around the orthodontic bracket with an unfilled sealant or a filled flowable composite restorative material. These researches used solutions that simulated a high cariogenic challenge *in vitro*. Although the results of these studies helped us better understand the preventive potential of these products, *in vitro* experimental conditions cannot deal with all the complexities of a living oral cariogenic environment. In the present study, under the *in situ* conditions used, only the CE group maintained similar hardness values to control groups. In the other experimental group (TE), the hardness values decreased when submitted to oral environment. Other researches with longer time exposure to oral environment and using a method capable of assessing the penetration, permeability, and degradation of the adhesives tested are necessary to verify the efficacy of these materials, mainly in patients with high caries risk.

Conclusions

Under the conditions of the study, the enamel impregnated with a bonding agent (Concise™) showed a significantly different result from other types of treatment evaluated. There was no recovery of microhardness when the enamel was etched with 35 per cent phosphoric acid and was exposed to the oral environment during 28 days. Moreover, none of the groups exposed to the intra-oral environment recovered completely, but the order of improvement was better in the CE group than the TE group, the latter being only slightly better than the EE group.

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