Effect of Bioactive Composites on Microhardness of Enamel Exposed to Carious Challenge

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ABSTRACT

This study verified if experimental composites containing calcium phosphate nanoparticles exert a protective effect against enamel demineralization. Three experimental resin-based composites containing 60 vol% of fillers were manipulated. Filler phase was constituted by silanized barium glass and 0%, 10% or 20% (by volume) of dicalcium phosphate dihydrate (DPCD) nanoparticles functionalized with the monomer triethylene glycol dimethacrylate (TEGDMA). Cavities (10 x 2 x 2 mm) were prepared in bovine enamel and restored using one of the experimental materials (n=10). Specimens were exposed to pH cycling (demineralizing solution: pH 5.0/4h, remineralizing solution: pH 7.0/20h, 14 days). Enamel Knoop microhardness (100g/10s) was measured on the surface (SH) and after transversal sectioning up to 90 µm depth (cross-sectional microhardness, CSH). Microhardness values and the percent of microhardness loss were analyzed ANOVA/Tukey test and Student's paired t-test (alpha: 5%). The materials did not differ in respect to SH. Enamel adjacent to DCPD-containing composite restorations showed smaller reductions in CSH (-1.2% to -3.5%) than the enamel from control group (-12.5%), while CSH of enamel restored with resin-modified glass ionomer was similar to the other groups (-4.5%). DCPD-containing composites reduced enamel demineralization in comparison to a conventional composite.

INTRODUCTION

Patients with high caries risk and teeth with a high number of restored surfaces are determinant factors in restoration survival and have shown to reduce the longevity of resin-based composite (RBC) restorations.[1-3] In order to reduce the incidence of recurrent caries lesions in restored teeth or slow down their progression, different strategies have been tested, including the addition of bioactive and/or antibacterial agents in the resin formulation.[4,5]

In vitro and *in situ* studies have demonstrated the remineralization potential of RBCs containing calcium orthophosphate particles (CaP).[6-9] CaP-containing composites offer protection against demineralization by acting as sources of Ca^{2+} and H_2PO-/HPO_4^{2-}, creating a supersaturated environment that favors remineralization.[10] Furthermore, Ca^{2+} stored in the dental biofilm can be released during pH drop events[9,11] and was also shown to increase fluoride retention.[12]

Recently, dicalcium phosphate dihydrate (DCPD) nanoparticles functionalized with triethylene glycol dimethacrylate (TEGDMA) were synthesized.[13] DCPD is a calcium orthophosphate phase that shows a relatively high solubility and refractive index similar to barium glass, which is important to maintain the resin composite optical behavior unaffected.[14] TEGDMA is a monomer frequently used in commercial resin-based composites and adhesive systems formulations. Its relative hydrophilicity allows water access to the DCPD nanoparticles, maintaining ion release at levels similar to those observed with non-functionalized particles.[14] Previous studies have shown that RBCs containing 20 vol% of TEGDMA-functionalized DCPD particles showed a flexural strength approximately 30% higher than those containing non-functionalized DCPD, which is important from the biomechanics standpoint.[14,15] Notwithstanding, in order not to over-compromise the material's mechanical properties, CaP content should be kept as low as possible without losing bioactivity.[16] Thus, it would be interesting to evaluate whether smaller fractions of DCPD are effective in protecting the enamel adjacent to the restoration against demineralization.

Based on the above, the objective of this study was to verify whether resin composites containing TEGDMA-functionalized DCPD particles in different concentrations would increase enamel resistance against demineralization caused by a cariogenic challenge *in vitro*. The null hypothesis tested was that enamel microhardness after pH cycling is not affected by the presence of DCPD in the RBC.

METHODS

COMPOSITE FORMULATION

Three composites were prepared with an organic phase constituted by an equimolar mixture of bisphenol-A glycidyl dimethacrylate (BisGMA) and TEGDMA, plus camphorquinone and ethyl-4-dimethylamino benzoate (EDMAB) as photoinitiators (0.5 wt% of each). Filler phase consisted of 0 vol% (control, DCPD_0), 10 vol% (DCPD_10) or 20 vol% (DCPD_20) of TEGDMA-functionalized DCPD particles plus silanated barium glass (D_{50}: 2 µm), totaling a 60 vol% filler load. Particles had 32 wt% of TEGDMA and a median size of 23 µm. Fillers were incorporated gradually and composites were mechanically mixed at 2500 RPM for 15s under vacuum (Speedmixer DAC 150.1 FVZ-K). Composites were not subjected to degassing and were kept refrigerated until 2 h before use.

SPECIMEN PREPARATION

Bovine enamel blocks (10 x 4 mm) were cut from the middle third of the buccal surface using a low-speed saw (IsoMet 1000), flattened and polished with silicon carbide abrasive papers (600-grit and 1200-grit, respectively), with a maximum enamel thickness removal of 300 µm. Enamel SH was assessed using a microhardness indenter (Shimadzu HMV 2) with a Knoop indenter. Five indentations were produced using a 100 g load for 10 s and averaged. The average KHN value for each block was used to assign them to one of the five experimental groups using stratified randomization (n=10).[17] Rectangular cavities (10 x 2 x 2 mm) were prepared using diamonds burs (F/G 837-016). For groups restored with the composites (i.e., DCPD_0, DCPD_10 and DCPD_20), a three-step etch-and-rinse adhesive was applied to the cavity walls according to the manufacturer's instructions (Adper Scotchbond Multipurpose Plus) and photoactivated for 20 s (1200 mW/cm^2, Bluephase N). Two layers of the experimental composites were applied incrementally into the cavity, each of them photoactivated for 20 s. A conventional glass ionomer cement (GIC, Fuji IX) was used to restore the cavities in the fourth group, also according to the manufacturer's instructions. Restorations were wet-polished with felt paper (1200-grit) for 1 min.

pH-CYCLING

A 1 mm-wide line was painted with nail polish in order to protect enamel from demineralization, creating a sound enamel area ("baseline") on both sides of the enamel surface *(Figure 1)*. Specimens were immersed in 66 ml (2.2 ml/mm^2) of demineralizing solution (DE: 1.28 mmol/L Ca, 0.74 mmol/L P, 0.03 ppm F in 0.05 mol/L acetate buffer, pH 5.0) for 4 h. Samples were rinsed and immersed in 44 ml (1.1 ml/mm^2) of remineralizing solution (RE: 1.5 mmol/L Ca, 0.9 mmol/L P, 150 mmol/L KCl, 0.05 ppm F in 0.1 M Tris buffer, pH 7.0) for 20 h, completing 24 h of pH cycling.[18] This procedure was repeated for 14 days and every four days the solutions were replaced for fresh ones.

SURFACE AND CROSS-SECTIONAL MICROHARDNESS

After pH-cycling, SH was measured. Three rows of five indentations were made, the first one located 10 µm from the composite/tooth interface and other two rows 50 µm apart from the adjacent indentations. At the baseline areas, after the careful removal of the protective nail polish with a scalpel blade, two rows of indentations were made 50 µm apart from each other *(Figure 2A)*. Then, the specimens were sectioned perpendicularly to the surface and polished as described before. Three columns (at 10, 60 and 110 µm from the composite/teeth interface) of five indentations 10, 30, 50, 70 and 90 µm from the enamel surface) were made on exposed enamel areas.[17] The mean values of all three indentations at each depth were calculated. For the baseline areas, one column of five indentations (same depths) was made *(Figure 2B)*.

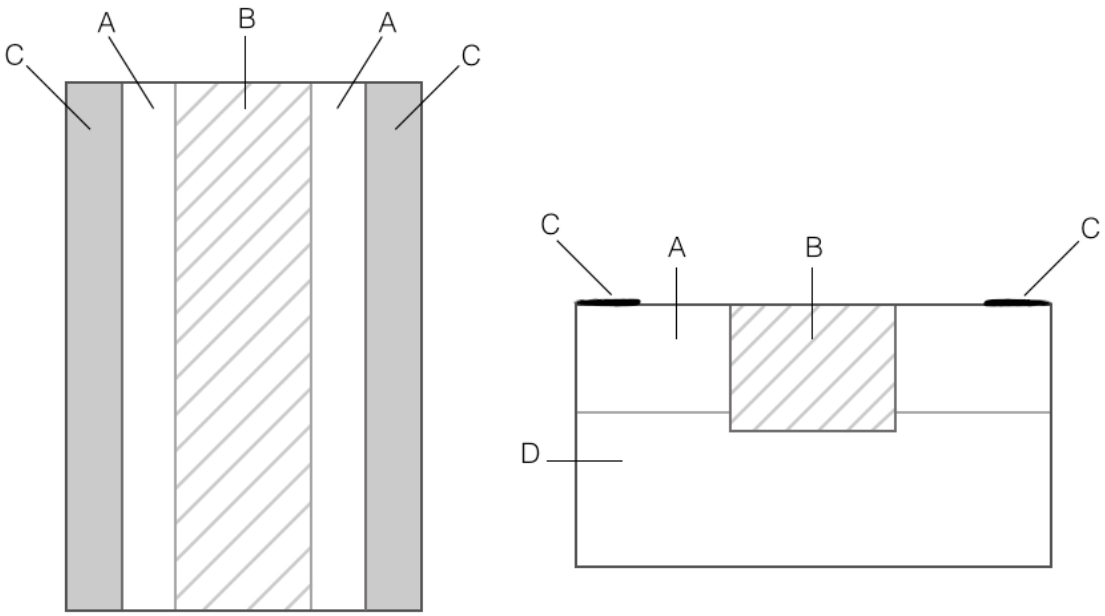

Figure 1: Schematic representation of the specimens used to determine SH and CSH. Left: Upper view, Right: Cross-sectional view. A: enamel area exposed to pH-cycling, B: restoration, C: enamel baseline area, D: dentin.

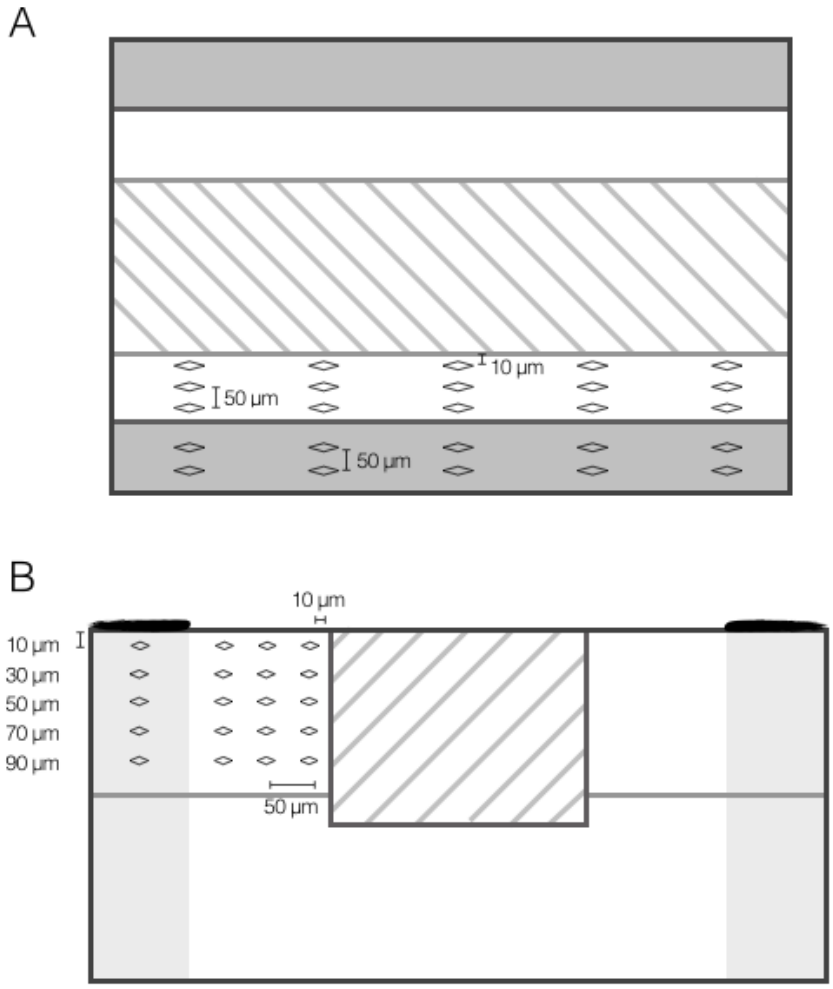

Figure 2: Diagram showing the position of the Knoop indentations on the specimen (A: upper view, B: cross-sectional view)

Variations in microhardness between baseline and exposed enamel (ΔKHN, %) were calculated according to the following equation:

$$\Delta KHN = \left(\frac{KHN_{exposed} - KHN_{baseline}}{KHN_{baseline}}\right) x100$$

STATISTICAL ANALYSIS

Statistical analysis was performed using MINITAB.[17] Data was checked for normality (Kolmogorov-Smirnov) and homoscedasticy (Levene). Surface microhardness (SH) at baseline and exposed areas, as well as its variation (in %) were subjected to one-way ANOVA/Tukey test (independent variable: restorative material). Additionally, for each experimental group, microhardness values on baseline and exposed enamel were compared by paired Student's t-test. Cross-sectional microhardness (CSH) for each experimental group was analyzed separately for baseline and exposed enamel using one-way ANOVA/Tukey test (independent variables: depth), while for each material and depth KHN of baseline and exposed enamel were compared by paired Student's t-test. CSH variation (in %) was analyzed using two-way ANOVA/Tukey test (independent variables: depth and restorative material). A significance level of 0.05 was pre-set for all analysis.

RESULTS

SURFACE MICROHARDNESS

Means and standard deviations for SH determined on the baseline and exposed enamel, as well as the variation between both are shown in Table 1. Results from the baseline area were statistically similar between all groups (p=0.737), as well as values from the exposed enamel (p=0.642). For all materials, pH-cycling produced significant reduction on enamel SH (p<0.001 for all groups except DCPD_10: p<0.01). SH reductions varied between -17% and -21% and no statistically significant differences were detected among groups (p = 0.733).

CROSS-SECTIONAL MICROHARDNESS

Means and standard deviations for CSH are shown in Figure 3. Groups restored with 10% or 20% DCPD resin composites did not show statistically significant differences in KHN between baseline and exposed enamel in any of the depths (Student paired t-test, p>0.05). Conversely, groups restored with the control composite and with GIC showed lower KHN values for the exposed enamel compared to baseline at some depths. Regarding CSH variation, ANOVA revealed that only "depth" and "restorative material" factors were statistically significant (p<0.01 for both), without interaction between them (p=0.061, Table 2). Considering the pooled means for the "restorative material" factor, the reduction in microhardness after pH cycling was statistically lower for teeth restored with DCPD-containing composites compared to the control composite. Teeth restored with GIC showed a reduction in microhardness statistically similar to all the other groups. The pooled means for the "depth" factor showed that CSH variation near the enamel surface (10 µm) was positive, i.e., there was an slight increase in enamel KHN. CSH variation recorded at 10 µm was statistically different than variations recorded at depths equal or greater than 50 µm.

Table 1. Mean and standard deviations of surface microhardness. Similar upper-case letters indicate absence of statistically significant differences between the groups in each column (ANOVA/Tukey test, p<0.05). Different lower-case letters indicate the presence of statistically significance difference between baseline and exposed ("after pH-cycling") for the same material (Student's t-test for paired samples, p<0.05)

Groups	Surface microhardness		Variation (%)*
	Baseline	After pH-cycling	
DCPD_0	308 (20) ^{Aa}	244 (24) ^{Ab}	-21 (8) ^{A}
DCPD_10	318 (51) ^{Aa}	256 (24) ^{Ab}	-18 (11) ^{A}
DCPD_20	305 (20) ^{Aa}	253 (10) ^{Ab}	-17 (5) ^{A}
RMGI	306 (16) ^{Aa}	251 (25) ^{Ab}	-17 (10) ^{A}

* negative values indicate reduction in enamel KHN after pH cycling.

DISCUSSION

In order to render bioactivity to current resin-based restorative materials, several research groups have tested the incorporation of CaP particles as ion-releasing fillers.[6-8,19-21] From a mechanical standpoint, the addition of CaP particles reduces the composite fracture strength, as CaP particles are not chemically bonded to the matrix.[22] In order to minimize the loss in mechanical properties, functionalization of these ion-releasing particles with TEGDMA has shown to promote a significant increase in the composite mechanical strength compared to the use of non-functionalized nanoparticles, without compromising ion release.[14]

SH reduction after a cariogenic challenge was similar among experimental groups. One possible hypothesis to explain this finding may be the pH cycling model used in this study.[18] Different from other pH cycling models,[6-8] both DE and RE solutions contained fluoride (<0.05 ppm), which may have masked the effect of the different restorative materials on the enamel surface microhardness.[23] The inability in detecting the effect of different materials at the early stages of the caries process constitutes a limitation of pH-cycling protocols, since they produce very shallow lesions.[24]

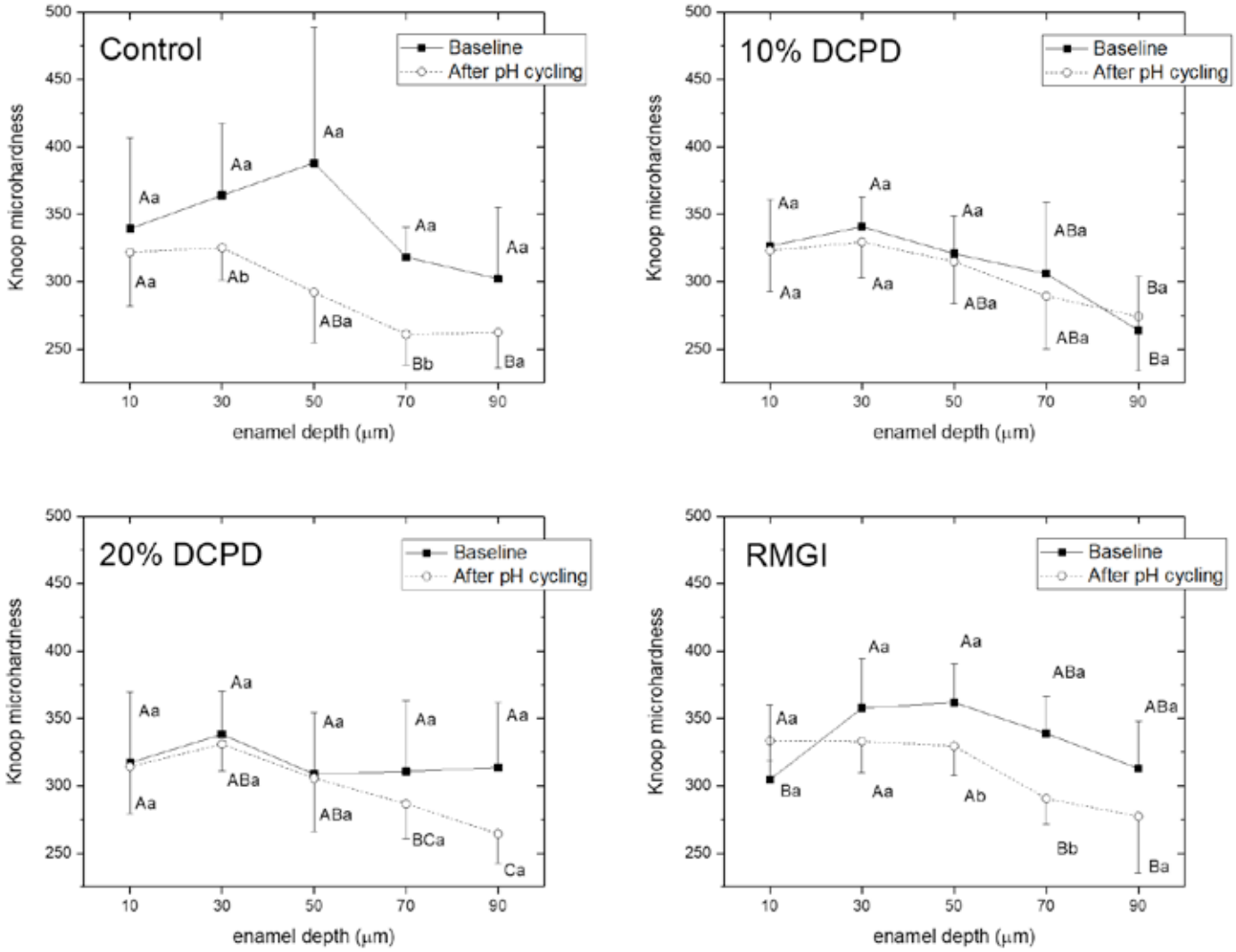

Figure 3: Mean and standard deviation of CSH as a function of depth and pH-cycling exposure for the four restorative materials tested. In each plot, similar upper-case letters indicate the lack of significant statistical differences between depths, for either baseline or exposed enamel (ANOVA/Tukey test, $p<0.05$) and similar lower-case letters indicate lack of statistically significant difference between baseline and after pH-cycling at a given depth (Student's paired t-test, $p<0.05$)

Table 2. Means and standard deviation of ΔKHN (%) of CSH, as a function of the restorative material and depth. Similar upper letters in columns and lower case letters in rows indicate the lack of significant statistical differences (two-way ANOVA, p<0.05)

Depth (μm)	Restorative Material				Pooled means*
	DCPD_0	DCPD_10	DCPD_20	RMGI	
10	-2.5 (18.2) ^{Aa}	-0.6 (8.8) ^{Aa}	1.2 (18.6) ^{Aa}	13.5 (25.2) ^{Aa}	2.9 (19.0) ^{A}
30	-9.1 (13.5) ^{Aa}	-3.0 (9.6) ^{Aa}	-1.5 (10.0) ^{Aa}	-6.2 (10.2) ^{ABa}	-5.0 (10.9) ^{AB}
50	-21.8 (14.8) ^{Ba}	-1.2 (13.1) ^{Aa}	0.2 (15.8) ^{Aa}	-8.5 (8.6) ^{ABa}	-7.8 (15.7) ^{B}
70	-17.5 (10.8) ^{ABa}	-4.6 (10.1) ^{Aa}	-5.8 (14.4) ^{Aa}	-13.6 (10.5) ^{Ba}	-10.3 (12.5) ^{B}
90	-11.8 (10.4) ^{Aa}	4.5 (10.2) ^{Aa}	-13.6 (17.7) ^{Aa}	-10.8 (14.5) ^{Ba}	-8.0 (15.2) ^{B}
Pooled means*	-12.5 (15.4) ^{b}	-1.2 (10.4) ^{a}	-3.5 (15.7) ^{a}	-4.5 (17.6) ^{ab}	

* positive values indicate increase in enamel KHN after pH cycling; negative values indicate decrease in enamel KHN after pH cycling.

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Moreover, it has been reported that CaP-containing composites have a more pronounced effect in the deeper areas of the lesion, whereas fluoride-releasing composites efficiently recovered mineral lost at the outer enamel.[7] Accordingly, in the present study teeth restored with GIC showed an increase in enamel CSH at 10 µm from the surface. However, at depths below 50 µm statistically significant reductions in CSH were recorded. For fluoride-releasing materials, a deeper penetration of fluoride into the lesion is dependent on higher F-gradients[25] and may further depend upon the nature of the lesion with respect to the initial porosity and depth.[23]

While the null hypothesis cannot be rejected for superficial microhardness, it was not confirmed for the transverse microhardness. In general, the enamel adjacent to composites containing DCPD showed lower reductions in CSH (pooled means) compared to the group restored with control material. Though the high scatering of data did not allow the detection of statistically significant differences between materials at a given depth, the numerical values suggested greater mineral loss in the control group compared to composites containing DCPD at 50 µm depth. Since, as mentioned above, high calcium phosphate contents negatively affect the mechanical properties of the material,[15,16] the finding that similar CSH variations were observed in teeth restored with either 10% or 20% DCPD-containing composites is relevant, as it suggests that the intended protective effect be achieved without a severe reduction in fracture strength.

Calcium and phosphate concentrations released by materials similar to those evaluated in this study were determined in a previous study.[15] Composites containing 10% DCPD released about 8 ppm calcium and 0.5 ppm dihydrogen phosphate, while composites containing 20% DCPD released 10 ppm and 1 ppm after 7 days (at pH 7.0). These values are higher than those reported by Melo *et al*.[9] for a composite containing 40% (by weight) of amorphous calcium phosphate after 28 days (4 ppm of calcium and <0.5 ppm of phosphate, pH 7). This can be explained by the greater solubility presented by the DCPD compared to the ACP.[26,27] It should be emphasized that ion release increases under acid pH conditions.[28] Thus, it is possible that the concentrations released in this *in vitro* study were higher than those previously reported.

Most of the available literature on CaP-containing materials deals with their remineralizing potential rather than a possible protective effect (i.e., demineralization inhibition). For example, sealants containing CaP and fluoride promoted increases in microhardness of artificial lesions between 24% to 35%.[25] In another study, a ACP-containing fissure sealant showed a 30% increase of SH of artificial caries lesions *in situ*.[29] One of the few studies that evaluated the protective effect of CaP-containing materials against demineralization showed a lower reduction of microhardness after a cariogenic challenge when a ACP-containing fissure sealant was used.[30] Similarly, an *in situ* study showed a lower mineral loss around the restoration made with a composite containing 40 wt% ACP compared to a conventional composite after 14 days in the presence of cariogenic biofilm. In addition, lesion depth adjacent to the ACP-containing composite was 59% lower than those observed for the conventional RBC.[9]

Teeth restored with GIC presented a CSH statistically similar to that of the other groups. Clearly, the pooled mean value displayed by the GIC group was influenced by the positive variation found at 10 µm. However, in deeper areas of the lesion the reductions in microhardness was numerically closer to that of the control group. This behavior can be explained by the mineral precipitation (fluoridated hydroxyapatite) on the enamel surface, constituted by ions coming from the DE and RE solution, as well as from the dissolution of the deeper enamel and the fluoride released by the material. These precipitates occlude the surface pores and limit the extent to which the remaining of the lesion may be repaired.[7,25,32] This process characterizes a mineral transfer in the dental structure, and not a mineral gain.[33]

In general, results from *in vitro* studies should be analyzed with caution, as they do not adequately reproduce the oral environment, characterized by the presence of salivary flow, acquired pellicule and biofilm.[34] Besides, pH-cycling occurs in a "closed" system, in which the mineral exchange between the enamel and the DE and RE solutions are limited by the ionic balance between them. Finally, though enamel microhardness varied between baseline and exposed areas, the formation of a subsurface caries-like lesion was not confirmed by transverse microradiography (TMR) analysis (not shown). Taking into account the limitations of this study, it can be stated that the DCPD-containing composites provided protection to the adjacent enamel exposed to pH-cycling, evidenced by the lower variation of CSH values in relation to the composite without DCPD.

MANUFACTURERS' DETAILS

- GIC, Fuji IX, GC Europe
- Bisphenol-A glycidyl dimethacrylate (BisGMA), triethylene glycol dimethacrylate (TEGDMA), camphorquinone and ethyl-4-dimethylamino benzoate (EDMAB) from Sigma-Aldrich, St Louis, MO, USA
- Silanated barium glass, FGM, SC, Brazil
- Speedmixer DAC 150.1 FVZ-K, FlackTek Inc., Landrum, SC, USA
- Isomet 1000, Buehler, Lake Bluff, IL, USA
- EcoMet 3000/AutoMet 2000, Buehler, Lake Bluff, IL, USA
- HMV 2, Shimadzu, Tokyo, Japan
- F/G 837-016, Microdont, SP, Brazil
- Adper Scotchbond Multipurpose Plus, 3M ESPE, St. Paul, MN, USA
- Bluephase N, Ivoclar Vivadent, Schaan, Liechtenstein
- Fuji IX, GC Europe, Leuven, Belgium
- Minitab 17, Minitab Inc., State College, PA, USA

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